

**The Characterisation Of Vasoactive Intestinal Peptide As A
Secretagogue In Human H295R Adrenocortical Cells**

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DECLARATION OF ORIGINALITY:

I declare that the composition of this thesis and the work presented herein is my own, unless otherwise stated.

Vanessa Jane Cobb.

DEDICATION:

I would like to dedicate this thesis to my family: Su, David, Adam and in memory of Mike, for their love, support and patience at all times.

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ABSTRACT

Although the main stimuli to cortisol and aldosterone secretion are ACTH and AII, respectively, increasing evidence has implicated other hormones and neurotransmitters in the regulation of steroidogenesis. In particular, nerve endings in close apposition to cortical cells and blood vessels have been demonstrated in humans (and other species) and a variety of neurotransmitters and neuropeptides detected within these nerves. Other work has established closer morphological interactions between cortical and medullary cells than hitherto suspected, with the possibility of paracrine interactions. Vasoactive intestinal peptide (VIP), which is present in adrenocortical nerves and chromaffin cells, has recently been studied as a potential steroidogenic agonist, but evidence for a direct effect on steroidogenesis in humans is controversial and very limited. The aims of this thesis were to investigate the direct action of VIP on adrenocortical steroid production, by using the human H295R adrenocortical carcinoma cell-line, and to determine the second messenger systems activated and the receptor types involved.

The steroid responses to AII and forskolin were first characterised in the H295R cell-line, including the effect of pretreatment with these agonists on the steroid phenotype of the cells. Forskolin-pretreatment directed steroidogenesis towards adrenal androgen production and away from 17-deoxysteroid production compared with AII-pretreated cells and control cells, demonstrating that the steroid phenotype of the cells could be modified by different agonists which utilise different second messenger systems.

VIP increased steroid secretion from H295R cells in a time-dependent manner. The pattern of steroid secretion was altered depending on the pretreatment conditions for the cell-line. VIP increased cortisol, corticosterone and androstenedione secretion from forskolin-pretreated cells but had no effect on androstenedione production from control cells or AII-pretreated cells. Although VIP increased cortisol and corticosterone from control and AII-pretreated cells, forskolin pretreatment enhanced the cortisol and corticosterone responses to VIP.

The effect of VIP on cortisol production from forskolin-pretreated H295R cells was studied further. VIP dose-dependently increased cortisol secretion (threshold dose $\approx 10^{-11}$ M, maximal dose $\approx 3.3 \times 10^{-8}$ M, $pEC_{50} = 9.2 (\pm 0.4)$ ($n=4$)). This response was accompanied by a parallel, dose-dependent increase in cAMP accumulation (threshold dose $\approx 10^{-11}$ M, maximal dose $\approx 3.3 \times 10^{-8}$ M, $pEC_{50} = 8.6 (\pm 0.5)$ ($n=4$)). Changes in total phosphoinositide turnover and cGMP production were not detected in response to VIP treatment of H295R cells.

A β -adrenoceptor-mediated mechanism for VIP-induced cortisol production was excluded. Comparison of dose-response curves demonstrated a similar potency of VIP and PACAP (a highly homologous peptide which shares some receptors with VIP) for cortisol secretion and cAMP production (pEC_{50} s for cortisol response to VIP and PACAP were $9.4 (\pm 0.4)$ and $9.8 (\pm 0.1)$, respectively ($n=3$) and the pEC_{50} s for cAMP response to VIP and PACAP were $8.7 (\pm 0.5)$ and $9.3 (\pm 0.1)$, respectively ($n=3$)), suggesting that these responses were mediated primarily by activation of VPAC receptors, not PAC1 receptors.

The VPAC2 receptor superagonist, RO-25-1553, failed to stimulate cortisol or cAMP production from H295R cells at doses of 10^{-9} M and 10^{-8} M ($P \geq 0.05$) whilst the VPAC1 receptor antagonists, Cl-Phe⁶, Leu¹⁷-VIP and acetyl-tyr-GRF-amide, had no effect on VIP-stimulated increases in cortisol and cAMP production from H295R cells ($P \geq 0.05$). Therefore, the VPAC receptor subtype involved in VIP-mediated cortisol and cAMP responses was not clearly defined using these pharmacological tools.

The technique of RT-PCR was used to detect the presence of VPAC1, VPAC2 and PAC1 receptor transcripts in H295R cells. VPAC1 receptor mRNA was detected in both untreated and forskolin-treated cells. VPAC2 receptor mRNA was not detected. A very low level of PAC1 receptor mRNA was observed. In conjunction with the pharmacological data obtained from comparison of VIP and PACAP dose-response curves, the weight of evidence is that the cortisol response to VIP is mediated predominantly by VPAC receptors, not PAC1 receptors.

Finally, the steroid responses of H295R cells to ANP, ATP, ACh, NA, 5HT and VP were briefly examined. ANP consistently inhibited steroid secretion in this cell-line.

In conclusion, VIP directly stimulates cortisol secretion from the human H295R adrenocortical cell-line by a mechanism involving activation of VPAC1 receptors coupled to the formation of cAMP. This supports the hypothesis that VIP could act directly as a physiological modulator of adrenocortical steroidogenesis in humans.

Abbreviations

AC	Adenylate cyclase
ACE	Angiotensin converting enzyme
ACh	Acetylcholine
AChE	Acetylcholinesterase
ACTH	Adrenocorticotrophic hormone
Adr	Adrenaline
AI	Angiotensin I
AII	Angiotensin II
AIII	Angiotensin III
ANP	Atrial natriuretic peptide / hormone
A-T-GRF-A	Acetyl-tyr-GRF-amide
ATP	Adenosine 5'-triphosphate
BSA	Bovine serum albumin
cAMP	Adenosine 3', 5' cyclic monophosphate
CASH	Cortical androgen stimulating hormone
cDNA	complementary deoxyribonucleic acid
CDP-DAG	Cytidine-diphosphodiacylglycerol
cGMP	Guanosine 3', 5' cyclic monophosphate
CGRP	Calcitonin gene-related peptide
CIP	Corticotropin-inhibiting peptide
CNS	Central nervous system
CPLV	Cl-Phe ⁶ , Leu ¹⁷ -VIP
CPM (cpm)	Counts per minute
CRH	Corticotrophin releasing hormone
CV	Coefficient of variation
DAG	1,2-Diacylglycerol
dbcAMP	Dibutyryl adenosine 3', 5' cyclic monophosphate
DBH	Dopamine β -hydroxylase
DBI	Diazepam binding inhibitor
DEPC	Diethylpyrocarbonate
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulphate
DMEM	Dulbecco's modified Eagle's medium

DNA	Deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DPM	Disintegrations per minute
EBSS	Earle's balanced salt solution
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethyleneglycol tetraacetic acid
Fskn	Forskolin
GC	Guanylate cyclase
GHRH	Growth hormone releasing hormone
G-protein	Guanine nucleotide-binding regulatory protein
H295R	NCI-H295R human adrenocortical tumour cell-line
12-HETE	12-Hydroxyeicosatetraenoic acid
HPA	Hypothalamic-pituitary-adrenal
3 β HSD	3 β -Hydroxysteroid dehydrogenase
11 β HSD	11 β -Hydroxysteroid dehydrogenase
5-HT	5-Hydroxytryptamine / serotonin
IBMX	Isobutyl-1-methylxanthine
IL-1	Interleukin-1
IL-6	Interleukin-6
Ins(4)P	Inositol 4-phosphate
Ins(1,4)P ₂	Inositol 1,4-bisphosphate
Ins(1,4,5)P ₃	Inositol 1,4,5-trisphosphate (IP ₃)
Ins(1,3,4,5)P ₄	Inositol 1,3,4,5-tetraphosphate
Ins(1,3,4,6)P ₄	Inositol 1,3,4,6-tetraphosphate
Ins(3,4,5,6)P ₄	Inositol 3,4,5,6-tetraphosphate
InsP ₅	Inositol pentakisphosphate
InsP ₆	Inositol hexakisphosphate
ITS	Insulin-transferrin-sodium selenite
IU/ml	International units per millilitre
JG	Juxtaglomerul
MDL	Minimum detection limit
MMLV-RT	Reverse transcriptase
mRNA	messenger ribonucleic acid
NA	Noradrenaline
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)

NO	Nitric oxide
NOS	Nitric oxide synthase
NPY	Neuropeptide Y
PACAP	Pituitary adenylate cyclase-activating polypeptide
PBR	Peripheral benzodiazepine receptor
PCR	Polymerase chain reaction
pGC	particulate guanylate cyclase
PGP 9.5	Protein gene product 9.5
PHI	Peptide histidine-isoleucine
PHM	Peptide histidine-methionine
PHV	Peptide histidine-valine
PI	Phosphoinositide
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol myristate acetate
PNMT	Phenylethanolamine N-methyltransferase
POMC	Pro-opiomelanocortin
PtdIns	Phosphatidylinositol
QC	Quality control
RAS	Renin-angiotensin system
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RR	Response ratio
RT-PCR	Reverse transcription-polymerase chain reaction
SAP	Steroidogenesis activator polypeptide
SCP2	Sterol carrier protein 2
s.d.	Standard deviation
SDS	Sodium dodecyl sulphate
sGC	soluble guanylate cyclase
StAR	Steroidogenic acute regulatory
TH	Tyrosine hydroxylase
TPA	12-O-tetradecanoylphorbol-13-acetate
UV	Ultraviolet
VIP	Vasoactive intestinal peptide

VP	Vasopressin
v/v	Volume per volume
w/v	Weight per volume
zf	Zona fasciculata
zfr	Zona fasciculata-reticularis
zg	Zona glomerulosa
zi	Zona intermedia
zr	Zona reticularis

1.0 Introduction

A general introduction to the adrenal gland, adrenal steroidogenesis and its regulation is presented in this chapter. In addition, the NCI-H295R (H295R) human adrenocortical carcinoma cell-line and vasoactive intestinal peptide (VIP) are introduced. More detailed introductions on sections of particular relevance to the studies presented in subsequent chapters are included at the beginning of the relevant chapter. For example, chapter 5.0 includes studies on the second messenger systems activated by VIP in H295R cells and an introduction to the second messenger systems studied in the chapter, as well as an introduction to the second messenger systems activated by VIP in other tissues, is included at the beginning of chapter 5.0 rather than in chapter 1.0. Sections of the thesis have been cross-referenced appropriately.

1.1 The adrenal gland

1.1.1 Gross anatomy of the adrenal gland

The adrenal glands comprise two ovoid organs situated on the superior pole of each kidney. The shape and size of the adrenal gland varies between species from almost spherical in the rat to more flattened and triangular in cattle. In the human, the left adrenal gland is crescent shaped whereas the right adrenal, lying close to the inferior vena cava, is more pyramidal. The paired human adrenals weigh approximately 8 g and measure about 4-6 cm in length, 2-3 cm in width and 1 cm in thickness. Each gland is surrounded by a thin layer of connective tissue and a thick fibrous capsule.

The organs are made up of two morphologically and functionally distinct types of glandular tissue; the inner catecholamine-producing medulla which arises from neuroectoderm and the outer steroid-secreting cortex of mesodermal origin. The medullary tissue comprises approximately 10 % of the weight of the human adrenal and is found situated mainly in the head of the adrenal gland with the cortical tissue in the tail. The body of the gland comprises both types of tissue.

The adrenal cortex consists of three roughly concentric zones; the outermost zona glomerulosa (zg), then the zona fasciculata (zf) and the inner zona reticularis (zr), which is in contact with the adrenal medulla. These zones are morphologically distinct and also functionally distinct in terms of which steroids are produced within the zones (section 1.3.1). The zf and zr, however, are often referred to as one functional zone (zfr). In some species, and especially in the human, there is a degree of convolution of the adrenal cortex, which results in close contact of both the zf and the outer zg with the central medulla in specific regions of the gland.

1.1.2 Adrenal blood supply

The adrenals are supplied with blood from small arteries branching from the aorta, the inferior phrenic artery and the renal artery. These arteries branch to form a subcapsular arteriolar plexus which subsequently distributes into two types of vessels; the thin-walled sinusoids which supply the cortex and the medullary arteries that convey blood directly to the medulla. In the rat, the subcapsular arterioles have muscular walls suggesting that they may be a site for the control of blood flow through the gland. The arterial blood flows through capillary loops surrounding the cells of the zg, then courses through radial capillaries extending through the zf, in a centripetal formation parallel to the columns of cells within the zf, before entering a further arterial plexus in the zr and finally draining into the central vein located within the medulla.

The human adrenal vasculature has the added complexity of the existence of arteriovenous loops, in which blood is carried through the zg and the zf then loops back to the exterior of the gland in a site adjacent to its origin. In addition, the central vein is surrounded by a cuff of inverted cortical tissue in which the three zones are clearly recognisable.

The number of medullary arteries varies between species, with a relatively large number in the dog and only 4-6 in the rat. These arteries pass through the cortex without branching, forming a network of capillaries within the medulla which then empty into the peripheral radicles of the central vein. The central vein empties

into the renal vein from the left adrenal and directly into the inferior vena cava from the right adrenal.

The vasculature of the adrenal is important in the functional zonation of the cortex and in the regulation of steroidogenesis (sections 1.1.2 and 1.6.3).

1.2 The adrenal medulla

The adrenal medulla consists almost entirely of chromaffin cells. These are irregularly shaped, polyhedral cells organised in cords or small clumps and surrounded by nerves, connective tissue and blood vessels. A distinguishing feature of the chromaffin cell is the inclusion of numerous chromaffin granules, containing the catecholamines, noradrenaline and adrenaline. In humans, the predominant catecholamine stored in the medullary chromaffin cells is adrenaline (85% of the catecholamine store) and the medulla contains distinct noradrenaline-containing and adrenaline-containing cells. In addition, a variety of non-catecholamine factors have also been identified in chromaffin cells, including VIP (Phillips & Pryde, 1987).

The adrenal medulla is innervated by cholinergic, preganglionic sympathetic neurons carried in the splanchnic nerve and stimulation of the splanchnic nerve results in the release of catecholamines from chromaffin cells. In addition, other factors, for example, VIP, appear to be released from chromaffin cells following splanchnic nerve stimulation (Ehrhart-Bornstein *et al*, 1991a, Bloom *et al*, 1987). The adrenal medulla also contains ganglion cells and post-ganglionic neurons. An intra-adrenal nerve plexus with cell bodies in the medulla, and nerve fibres in the cortex, has also been described (Holzwarth *et al*, 1987) (section 1.6.4.1). This intra-adrenal nerve system contains several neural agents including noradrenaline, neuropeptide Y (NPY) and VIP (Engeland *et al*, 1998, for review) (section 1.6.4.1).

1.3 The adrenal cortex

1.3.1 Adrenocortical Zonation

The adrenal cortex is divided into three roughly concentric zones, the outermost zg, the zf and the innermost zr. Initially these zones were distinguished morphologically but a functional division has been shown to underlie these morphological differences (see *Figure 1.1* for a diagrammatic representation of adrenocortical zonation). The zg lies just below the capsule of the gland and constitutes approximately 15 % of the cortex in the human. The cells are small with a low cytoplasm / nucleus ratio and large mitochondria with shelf-like cristae. They are characteristically arranged in whorls, loops or baskets about 4-6 cells in diameter, though the volume of the zg alters in response to the physiological status of the animal.

The cells of the zf are larger than those of the zg, have a higher cytoplasm / nucleus ratio, small ovoid mitochondria with tubulovesicular cristae and a lipid-rich cytoplasm. They comprise approximately 75 % of the cortex and are arranged as a series of centripetally orientated cords.

The network arrangement of cells of the human zr is sharply demarcated from both the zf and the medulla and comprises cells of intermediate size and cytoplasm / nucleus ratio. The cytoplasm is lipid-poor and contains numerous lipofuscin granules. The degree of demarcation of the three zones varies between species; for example, the zf and zr of the bovine adrenal cortex merge to form a single layer, often referred to as the zfr, whilst the bovine zg is clearly defined from the inner zones. In contrast, the human zg is poorly demarcated and cells from the zf can be observed directly under the capsule.

Additional zones may be observed in some species. These include 1) the X-zone (Chester-Jones *et al*, 1957), found between the zr and medulla in mice. The X-zone involutes at puberty to form a band of connective tissue; 2) the special zone, found only in the inner cortex of adult female marsupials; 3) the zona intermedia (zi), found in rat, cattle and sheep, consisting of a layer 2-3 cells deep and situated

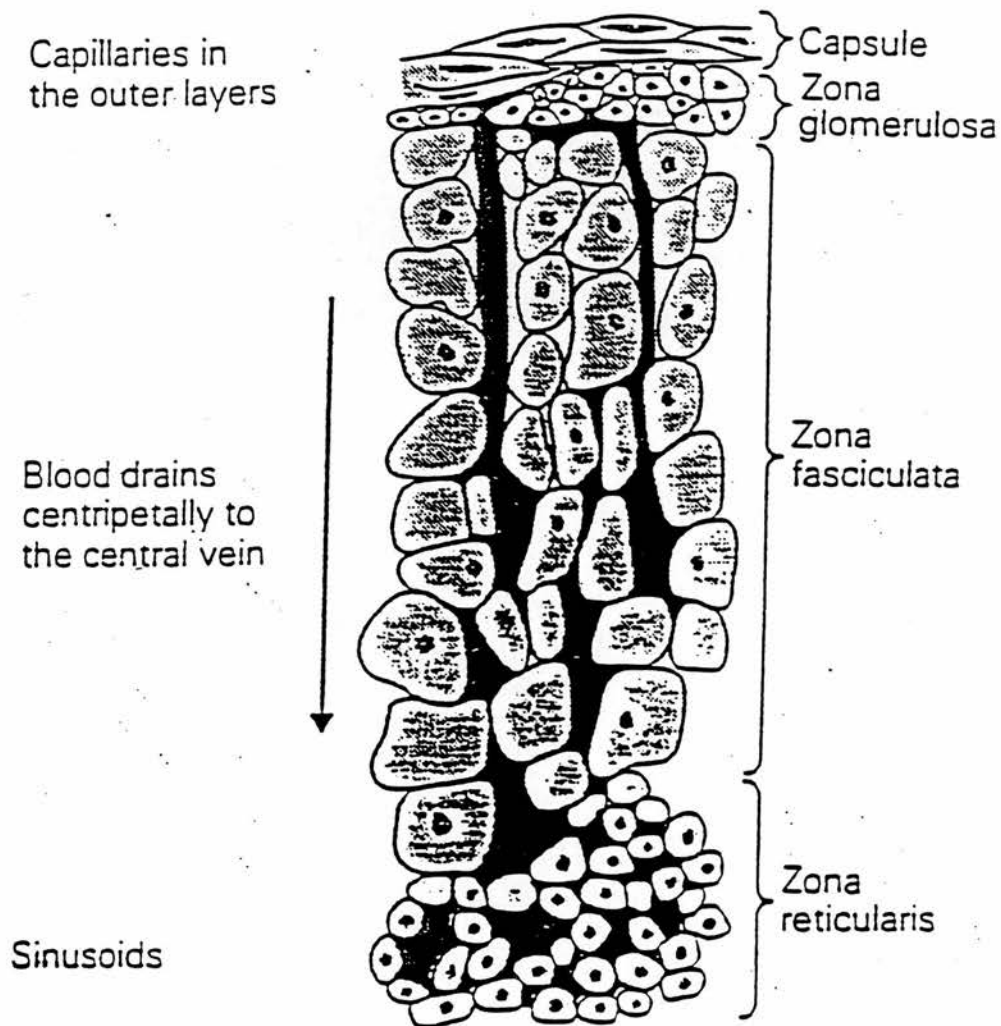


Figure 1.1

Zonation of the mammalian adrenal cortex. Diagrammatic representation of the arrangement of cells in a cross-section of the adrenal gland.

between the zg and zf (Deane, 1962); 4) the fetal zone, found in the fetal adrenal of primates, situated between the definitive cortex and the medulla. The fetal zone constitutes the majority of the fetal adrenal mass but degenerates rapidly after birth and has completely disappeared by one year.

Functional zonation of the adrenal is defined by the differences in enzyme complements between zones and the resultant differences in steroids produced by each zone. It closely approximates to the morphological zonation in that the zg is the only site of aldosterone production whereas the zfr mainly produces cortisol (corticosterone in rats) and adrenal androgens are synthesised principally by cells of the zr.

Several hypotheses exist to explain the formation and maintenance of the adrenocortical zones, including the “zonal hypothesis”, the “cell migration hypothesis” and the “transformation field hypothesis”.

1.3.1.1 The Zonal Hypothesis

The “zonal hypothesis” suggests that each zone is self-contained, responsible for its own replenishment of cells following local cell death (Chester Jones, 1948). It was based on the finding that all three zones contain proliferating cells. The zonal theory proposes that each zone contains differentiated cells with its fixed complement of steroidogenic enzymes.

1.3.1.2 The Cell Migration Hypothesis

The “cell migration hypothesis” proposes that a stem cell layer exists beneath the capsule which contributes cells to the zg. These cells then migrate through each of the zones, changing steroidogenic function in the process, and then dying within the zr (Zajicek *et al*, 1986).

Variations on the cell migration hypothesis include the suggestion by Belloni *et al* (1978) that the stem cell layer exists as an intermediate zone between the zg and

zf, with cells migrating first into the zg then looping back towards and through the zf and zr. Mitani *et al* (1994) identified an intermediate zone, 5-10 cells thick, between the rat zg and zf. These cells were incapable of aldosterone and corticosterone synthesis because they lacked P450aldo and P450c11 β . Pulse-chase experiments involving 5-bromo-2'-deoxyuridine labelling demonstrated that cells from this layer migrate to the zfr, suggesting that this may be the progenitor cell zone in the rat adrenal cortex (Mitani *et al*, 1994).

1.3.1.3 The Transformation Field Hypothesis

This hypothesis suggests that the width of the zf alters at the expense of the widths of the other two zones, in particular the zg. For example, ACTH treatment results in an increase in zf width with a concomitant decrease in the width of the zg (Bachmann, 1954).

1.3.1.4 The Steroid Gradient Hypothesis

The steroid gradient hypothesis proposed by Hornsby (reviewed by Hornsby in *The Adrenal Cortex*, edited by Anderson & Winter, 1985), argues that the centripetal vascular system creates a gradient of a particular substance across the cortex (suggested by Hornsby to be corticosterone) which regulates the expression of the steroidogenic enzymes. The enzymes proposed to be sensitive to this form of regulation include aldosterone synthase, 11 β -hydroxylase and 21-hydroxylase. This hypothesis attempts to explain the phenomenon of functional zonation and is compatible with the cell migration theory of cell replenishment of the zones. The hypothesis is dependent on the concept that the cells in each zone arise from the same basic cell type, exhibiting temporary functional and morphological differences. This concept is supported by the finding that cultured adrenocortical cells from each of the zones quickly revert to the same steroidogenic phenotype (Hornsby & Crivello, 1983).

It now appears that aspects of all the hypotheses described (1.1.3.1 - 1.1.3.4) may be reconciled within the adrenal (discussed by Wolkersdorfer & Bornstein, 1998). Recent studies of apoptosis (programmed cell death) markers and proliferation markers have indicated that cells in all three zones undergo apoptosis to differing degrees (Wolkersdorfer *et al*, 1996). In addition, proliferation and DNA repair occurs in zones of expected cell senescence, that is, the zr. Thus, it has been hypothesised that tissue remodelling in the adrenal may be regulated at several different levels throughout the gland and within each zone (Wolkersdorfer & Bornstein, 1998).

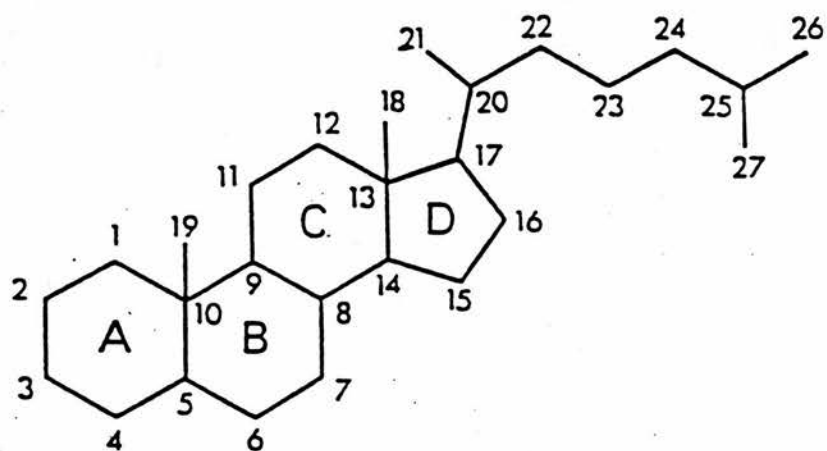
1.4 Adrenocortical Steroids

The adrenal cortex produces three main types of steroids: mineralocorticoids (for example, aldosterone), glucocorticoids (for example, cortisol) and adrenal androgens (for example, androstenedione and dehydroepiandrosterone (DHEA)). The precursor to steroid production is cholesterol, a 27 carbon molecule consisting of three cyclohexane rings (A, B and C) and one cyclopentane ring (D). The structure of an unnatural compound, cholestane, which can be considered to represent the backbone of the steroid structure, is illustrated in *Figure 1.2*.

1.4.1 Mineralocorticoids

The major mineralocorticoid produced by the adrenal cortex is aldosterone, a 21 carbon molecule with an 11 β -hydroxyl group which usually exists in a hemiacetal form with the aldehyde group at C18.

Formation of aldosterone is dependent on the presence of the enzyme P450aldo (aldosterone synthase). This enzyme is only present in the zg. The pathway leading to aldosterone production is illustrated in *Figure 1.3*. As can be seen, corticosterone is produced by this pathway and is a precursor to aldosterone synthesis in cells containing P450aldo.



Cholestane

Figure 1.2

The basic steroid structure. Cholestane, which has 27 carbon atoms in the positions shown, is not found in nature, but may be considered chemically as the parent hydrocarbon for cholesterol and related molecules.

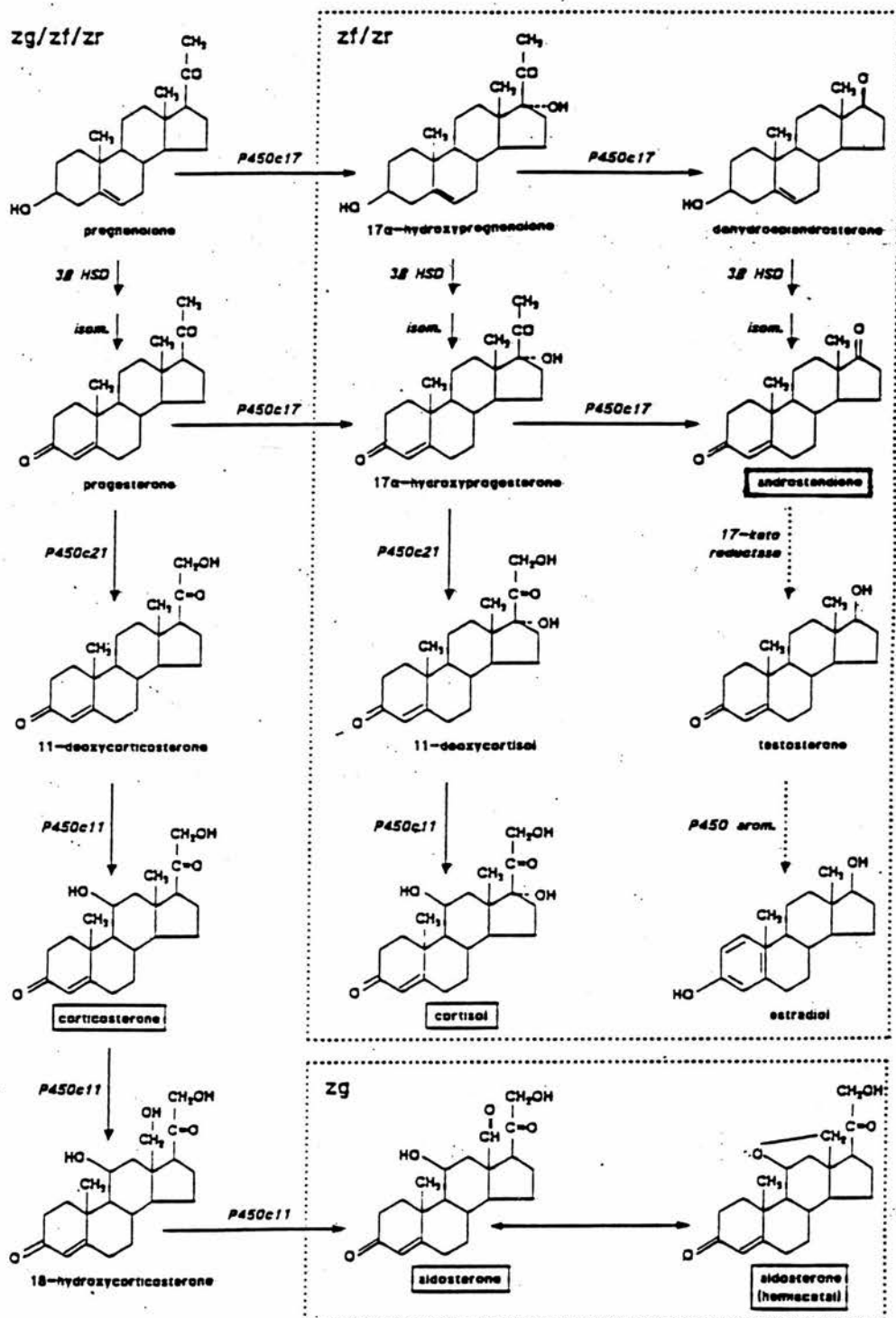


Figure 1.3

Major pathways of adrenocortical steroidogenesis in mammals. Steroids in boxes (solid line) are the steroids measured in these studies. Reactions confined to particular adrenocortical zones are enclosed in dotted boxes.

The major action of aldosterone is on the distal convoluted tubule of the kidney where it regulates Na^+ and K^+ homeostasis by promoting Na^+ reabsorption and K^+ , H^+ and NH_4^+ excretion. Similar actions on Na^+ / K^+ exchange in other secretory epithelia, such as the intestinal mucosa, have been demonstrated. Aldosterone is released in response to hypovolaemic stress. Thus, altered electrolyte and fluid status is the most important physiological stimulus to aldosterone secretion and can be induced experimentally by dietary sodium restriction or reduction of extracellular fluid volume, for example, by controlled haemorrhage. Physiological stimulation of aldosterone production within the adrenal is mediated primarily by AII and increased K^+ . ACTH can also increase aldosterone production (Ganguly & Davis, 1994).

1.4.2 Glucocorticoids

Glucocorticoids are produced primarily by the zfr and the major glucocorticoid in the human, bovine, sheep and guinea-pig is cortisol. The pathway for cortisol synthesis is illustrated in *Figure 1.3*. Cortisol and corticosterone have similar structures (see *Figure 1.3*), the only difference being an additional 17α -hydroxyl group in cortisol. The presence of the steroidogenic enzyme P450c17 (17α -hydroxylase) is a requirement for cortisol production and the lack of this enzyme in most rodent adrenals means that corticosterone is the major glucocorticoid secreted in these species.

Glucocorticoids have a diverse array of actions including effects on carbohydrate and protein metabolism. In general, these actions are seen to be in opposition to those of insulin in that glucocorticoids raise blood glucose levels by stimulating increased hepatic gluconeogenesis and increased protein catabolism, to release amino acids which are mobilised to the liver, for use in liver protein synthesis and gluconeogenesis (Brooks, 1979).

Glucocorticoids also have an anti-inflammatory action in response to injury or infection by inhibition of cytokine production (Bateman *et al*, 1989). Interactions between the immune system and glucocorticoid secretion exist, with certain

cytokines such as IL-1 and IL-6 stimulating glucocorticoid production and glucocorticoids acting as immunosuppressants (Bateman *et al*, 1989).

Excessive amounts of renal cortisol due to inhibition of 11 β -hydroxysteroid dehydrogenase (11 β -HSD), result in aldosterone-like actions on Na⁺ and water retention due to an action on mineralocorticoid receptors (Edwards & Stewart, 1991; Funder, 1996). Corticosterone is a less potent glucocorticoid than cortisol in terms of gluconeogenesis and anti-inflammatory action, but has a greater sodium-retaining activity (Brooks, 1979).

The major physiological stimulus for glucocorticoid production at the site of the adrenocortical cell is ACTH.

1.4.3 Adrenal androgens

The main adrenal androgens, androstenedione, DHEA and 11 β -hydroxyandrostenedione, are secreted predominantly by the zr in humans and guinea-pigs but by both the zf and zr in rats. The sulphated form of DHEA (DHEAS) is produced in large quantities by the human adrenal.

This group of steroids are 19 carbon molecules consisting of the 4 carbon rings (A, B, C and D) but no side chain (see *Figure 1.3* for structures and biosynthesis pathway). They are relatively weak androgens compared with testosterone but can be secreted in large amounts after adrenarche. They are further converted to the potent androgen, testosterone, or to oestrogens in other tissues.

Stimuli involved in androgen production are still unclearly defined. The main debate is whether androgen production is stimulated predominantly by ACTH (Anderson, 1980) or whether some other substance, often termed CASH (cortical androgen stimulating hormone), regulates adrenal androgen production, either in conjunction with or independently of ACTH. Indications that ACTH is not the only major regulator of adrenal androgen secretion arise from a number of physiologic and pathologic situations in which a divergence of adrenal androgen production and ACTH secretion, either measured directly or as reflected by cortisol concentrations,

exists (McKenna & Cunningham, 1991; Parker, 1991 (review)). These include the phenomenon of adrenarche, where adrenal androgen production increases, at about age 5 years, reaching a peak around puberty, remaining steady until about age 40 / 50 years and thereafter declining. At the same time, ACTH and cortisol levels remain relatively unaltered.

Candidates for CASH have included the POMC peptide, 'Joining Peptide' (POMC-(79-96)) (Clarke *et al*, 1996), though this has been disputed (Penhoat *et al*, 1991; Robinson *et al*, 1991), β -endorphin (Clarke *et al*, 1996) and prolactin (Higuchi *et al*, 1984).

1.5 Adrenocortical Steroid Biosynthesis

The steroid pathways leading to synthesis of these main groups of adrenal steroids are illustrated in *Figure 1.3*. Cholesterol is either taken up into adrenocortical cells from the circulation or synthesised de novo from acetate and is stored within the cell as cholesteryl ester droplets. Stimulation of steroidogenesis, for example by ACTH, leads to the conversion of cholesterol esters to free cholesterol and translocation to the mitochondria where conversion to the 21-hydrocarbon, pregnenolone, takes place (sections 3.1.1.1 and 1.5.1.1).

Two major types of enzymes are involved in steroid biosynthesis: 1) cytochrome P450s and 2) steroid dehydrogenases. The main enzymes involved in human adrenal steroid biosynthesis and the genes encoding them are listed in *Table 1.1*). These enzymes are described in more detail in section 1.5.1.

1.5.1 Adrenocortical Steroidogenic Enzymes

The cytochrome P450 enzymes are found in most tissues and are located in the mitochondria and endoplasmic reticulum. The active site of each enzyme contains a heme group which binds the substrate. Steroidogenic P450s catalyse either a single hydroxylation at a specific position on the steroid or a series of consecutive hydroxylations which result in C-C bond cleavage or aromatisation of

Characteristics of human adrenal cytochrome P450 genes

Enzyme	Location	Genes (n)	Chromosome	Protein (aa)
P450scc	mitochondrial	1	15	482
P450c11	mitochondrial	1	8	479
P450c18/aldo	mitochondrial	1	8	479
P450c17	microsomal	1	10	508
P450c21	microsomal	1 (1)	6	494-495

Table 1.1

Genes encoding the major human steroidogenic enzymes of the adrenal cortex. Numbers in brackets represent pseudogenes. For review see Hanukoglu (1992).

the steroid ring (Hanukoglu *et al*, 1992). The process of hydroxylation involves transfer of electrons from NADPH via a series of electron carriers, such as adrenodoxin, to P450 oxidase, which monooxidises the P450 enzyme using O₂. In the mitochondria, electrons derived from NADPH are transferred to the iron-sulphur protein, adrenodoxin, by the FAD-containing adrenodoxin reductase. The reduced adrenodoxin dissociates from the adrenodoxin reductase and forms a complex with cytochrome P450, to which the steroid substrate is bound. After reduction of P450, the oxidised adrenodoxin returns to the adrenodoxin reductase. In the microsomes, a single FMN and FAD containing reductase transfers electrons between NADPH and cytochrome P450.

1.5.1.1 P450scc (cholesterol side-chain cleavage enzyme)

Transfer of cholesterol across the inner mitochondrial membrane may limit steroid flux overall. However, P450scc is the rate-limiting enzyme of steroid biosynthesis, catalysing the conversion of the 27-hydrocarbon cholesterol to the 21-hydrocarbon pregnenolone via three successive monooxygenations (C20 hydroxylation, then C22 hydroxylation and finally cleavage of the C20-C22 bond). This step occurs at the start of all three adrenocortical steroidogenic pathways.

The enzyme exists as a complex with adrenodoxin and adrenodoxin reductase, which act as electron transport proteins transferring electrons from NADPH to the terminal oxidase, P450scc (section 1.5.1). The enzyme has been cloned in the human, bovine and rat species.

In the adrenal cortex, P450scc has been localised to the inner mitochondrial membrane and is present in all three zones of the cortex (reviewed by Ishimura & Fujita, 1997).

1.5.1.2 3 β HSD (3 β -hydroxysteroid dehydrogenase)

Pregnenolone and 17 α -hydroxypregnenolone (synthesised from pregnenolone by 17 α -hydroxylase (see 1.5.1.3) are converted to progesterone and 17 α -

hydroxyprogesterone, respectively, via the action of 3 β HSD. This enzyme catalyses a two step reaction: dehydrogenation of 3 β -hydroxy-5-ene-steroid and isomerisation of 3-oxo-5-ene-steroid.

At least two types of 3 β HSD have been cloned in the human, though only the type II enzyme has been detected in the adrenal cortex (reviewed in Labrie *et al*, 1994).

3 β HSD has been localised in the same cells as P450_{scc}, in all three zones of the adrenal cortex and appears to be situated in the membrane of the smooth endoplasmic reticulum (Ishimura & Fujita, 1997 for review).

1.5.1.3 P450c17 (17 α -hydroxylase / 17, 20 lyase)

This enzyme catalyses two separate reactions: 1) 17 α -hydroxylation of 21-hydrocarbon steroids and 2) cleavage of the C17-C20 bond of 21-hydrocarbon steroids. The first reaction is involved in glucocorticoid and mineralocorticoid synthesis and comprises the conversion of pregnenolone and progesterone to 17 α -hydroxypregnenolone and 17 α -hydroxyprogesterone, respectively. The second reaction is essential for the biosynthesis of androgens and results in formation of DHEA from 17 α -hydroxypregnenolone (and also formation of androstenedione from 17 α -hydroxyprogesterone in the rat, but not the human) (see *Figure 1.3*) (Hanukoglu *et al*, 1992 for review).

The regulation of the ratio of 17 α -hydroxylase activity / 17, 20 lyase activity is still unclear. It has been proposed that lyase activity is dependent on the rate of flow of electrons to P450c17. For example, an increased molar ratio of the transfer protein P450 oxidoreductase to P450c17 would result in an increased ratio of lyase activity to 17 α -hydroxylase activity of P450c17. Alternatively, a cAMP-dependent phosphorylation of serine and threonine residues on P450c17 has been shown to be important for lyase activity, possibly by increasing the affinity of P450c17 for redox partners (reviewed by Miller *et al*, 1997).

The enzyme has been immunocytochemically localised to the smooth endoplasmic reticulum of cells of the zf and zr, with strongest staining in the zf and no staining in the zg (reviewed by Ishimura & Fujita, 1997). This is in accordance with the absence of cortisol production in the zg.

Only one gene for P450c17 has been identified in the human whereas cattle appear to have at least three CYP (P450c17) genes (Miller *et al*, 1997 for review). P450c17 is absent from most rodent adrenals and, consequently, these species utilise corticosterone as their major glucocorticoid.

1.5.1.4 P450c21 (21-hydroxylase)

This microsomal enzyme catalyses the formation of the precursors of cortisol and corticosterone, namely deoxycortisol and deoxycorticosterone, from 17 α -hydroxy progesterone and progesterone, respectively (*Figure 1.3*). It is expressed in all three zones of the cortex (Ishimura & Fujita, 1997). Since P450c21 is required for both mineralocorticoid and glucocorticoid synthesis, a deficiency in the 21-hydroxylation step channels steroid biosynthesis in the direction of androgen production. For example, in the condition of congenital adrenal hyperplasia with 21-hydroxylase deficiency, the cortex is broadened and consists mainly of zr-type cells. Excessive production of 17 α -hydroxylated substrates for 17, 20-lyase results in heightened output of C19 steroids, DHEA and androstenedione. Androstenedione may be converted peripherally to testosterone, resulting in virilisation.

1.5.1.5 P45011 β (11 β -hydroxylase)

P45011 β is responsible for the formation of cortisol from deoxycortisol and corticosterone from deoxycorticosterone. Like P450scc, P45011 β exists as a complex with adrenodoxin and adrenodoxin reductase.

The enzyme has been localised to the inner mitochondrial membrane of cells from all three adrenocortical zones in the bovine adrenal, though higher levels are generally found in the zfr. The bovine enzyme exists as two similar isoforms, one

present in the zg and the other in the zfr (White *et al*, 1992, for review). The zg isoform catalyses aldosterone formation via 11 β -hydroxylation of deoxycorticosterone, followed by 18-hydroxylation and 18-oxidation. The 18-hydroxylation and 18-oxidation activities of the zfr isoform are suppressed, preventing aldosterone formation in the zfr.

In the rat adrenal, however, P45011 β appears to be localised exclusively in the zfr (Ishimura & Fujita, 1997). Thus, the rat zg only synthesises relatively low amounts of corticosterone through the action of P450aldo. A distinct enzyme, encoded by a separate gene, with 11 β -hydroxylation, 18-hydroxylation and 18-oxidation activities exists in the rat and human zg, which is responsible for aldosterone synthesis.

P45011 β is also capable of hydroxylating androstenedione resulting in the formation of 11 β -hydroxylated androgens.

1.5.1.6 P450aldo (aldosterone synthase)

Aldosterone synthase (also termed P450cmo, P450c18 or P450aldo) catalyses the 11 β -hydroxylation and 18-aldehyde conversion of deoxycorticosterone to aldosterone (reviewed by White *et al*, 1992). The enzyme has some properties similar to P45011 β but is encoded by a separate gene (CYP11B2 gene for P450aldo compared with CYP11B1 gene for P45011 β). The human gene for aldosterone synthase has been isolated (Mornet *et al*, 1989) and transcription of the gene is regulated by AII (White *et al*, 1992). It has been localised to the mitochondria of rat and hamster zg cells (reviewed by Ishimura & Fujita, 1997).

1.6 Regulation of steroidogenesis

Steroid hormone output is regulated at several levels including:

- 1) *mass of the zone* in which the steroid is synthesised; for example, an increase in zone mass contributes to an increase in cortical aldosterone output relative to other end-pathway steroids.
- 2) *substrate availability*; especially the rate at which cholesterol is transported to P450_{scc}, located on the inner mitochondrial membrane.
- 3) *enzyme activity*; determined by factors such as post-translational modification and the availability of co-factors.
- 4) *levels of steroidogenic enzymes*; determined by rates of gene transcription, stability of mRNA and translation rates. An increase in all adrenal steroidogenic enzymes will lead to a general increase in steroid production, subject to constraints imposed by rate-limiting steps, with no alteration in the secretion ratios of one end-pathway steroid to another (the adrenal end-pathway steroids being aldosterone, cortisol (or corticosterone in the rat) and androstenedione (or DHEA) in this context. An increase in the relative amount(s) of enzyme(s) will increase the overall production of steroids on that enzyme pathway as well as the ratio of the different end-pathway steroids.

Each of these parameters can be regulated together or separately by steroidogenic agonists, for example, ACTH and AII.

1.6.1 The Hypothalamic-Pituitary-Adrenal (HPA) axis

Secretion of glucocorticoids from the adrenal cortex is stimulated, in part, by adrenocorticotrophic hormone (ACTH). This 39 amino acid peptide is synthesised in the anterior pituitary from a precursor molecule, pro-opiomelanocortin (POMC), from which other peptides are also derived such as β -endorphin.

Stimulation of ACTH synthesis and secretion is primarily under the control of corticotrophin releasing hormone (CRH), which is a 41 amino acid hypothalamic peptide synthesised in the parvocellular portion of the paraventricular nucleus.

Release of CRH from axon terminals at the median eminence occurs in response to stress, such as pain, fear, hypoglycaemia, hypoxia and haemorrhage. The CRH enters the hypophyseal portal blood system and is transported to the anterior pituitary where it stimulates receptors on pituitary corticotrophs to synthesise and secrete ACTH (see *Figure 1.4* for illustration of the HPA axis).

ACTH is transported humorally to the adrenal where it acts on ACTH receptors to stimulate glucocorticoid production from the zf. A feedback inhibition mechanism exerted by the glucocorticoids regulates the release of ACTH and CRH at both the pituitary and hypothalamic levels.

Other factors such as arginine vasopressin (VP) and catecholamines can indirectly regulate steroidogenesis through the HPA axis. The most important of these is VP, which is synthesised in the hypothalamus and acts in the pituitary to potentiate the action of CRH.

Release of ACTH and glucocorticoids throughout the day follows a circadian rhythm. The highest levels in human plasma are found just before waking and the lowest levels in the late evening before sleeping. Glucocorticoids exert a feedback inhibition on the circadian rhythm of the HPA axis.

1.6.2 The Renin-Angiotensin System (RAS)

The RAS is involved in the maintenance of volume homeostasis and electrolyte balance and is activated in conditions such as haemorrhage and sodium depletion. A decrease in sodium filtration in the kidney results in the release of renin, a 38 kDa proteolytic enzyme, from the juxtaglomerular (JG) cells in the afferent arteriole of the kidney (Peach, 1977). Release of renin is also stimulated by decreased renal perfusion pressure and sympathetic stimulation of JG cells. This enzyme is released into the circulation where it converts the plasma protein angiotensinogen to the decapeptide angiotensin I (AI). The relatively inactive AI is then cleaved by angiotensin converting enzyme (ACE), found primarily in the lungs, to form the biologically active octapeptide angiotensin II (AII) (Vallotton *et al*,

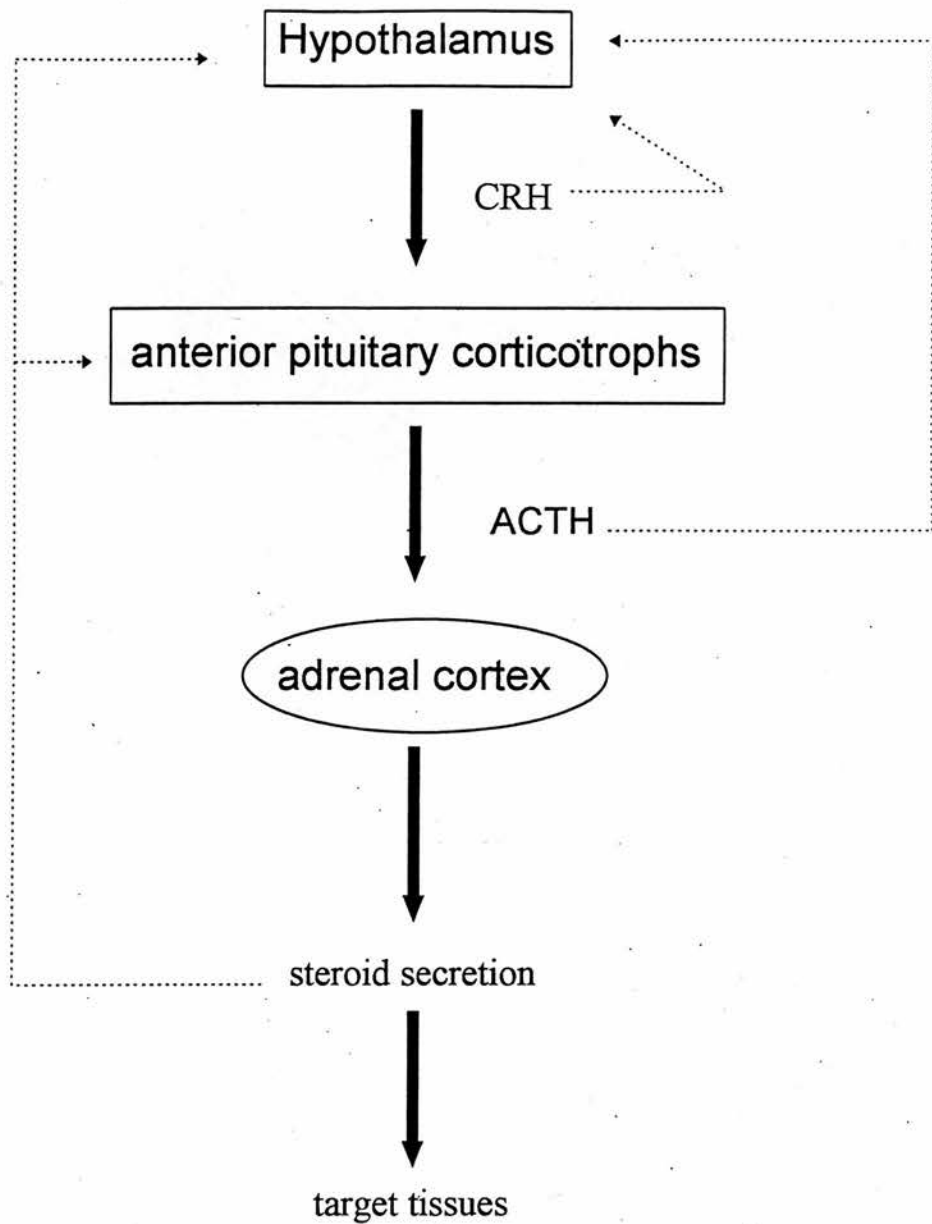


Figure 1.4

Simplified illustration of the HPA axis. Stimulatory pathways are indicated by solid lines and inhibitory pathways are indicate by dotted lines.

1987). AII exerts a negative feedback inhibition on renin release from JG cells (see *Figure 1.5* for diagrammatic representation of the systemic RAS).

AII exerts a variety of actions involved in the maintenance or restoration of plasma volume, blood pressure and body sodium content, including vasoconstriction and stimulation of aldosterone production from the adrenal cortex (Vallotton *et al*, 1987).

AII has a short half-life in plasma (2-3 mins) and is rapidly broken down to shorter fragments by aminopeptidases. Aminopeptidase A cleaves AII to form the heptapeptide AIII (des-Asp¹-AII), which is physiologically active and is actually more potent than AII in the stimulation of aldosterone release (Peach, 1977).

1.6.3 Importance of the adrenal vasculature in the regulation of steroid secretion

Increased blood flow through the adrenal has been associated with increased steroidogenesis, a phenomenon attributed to several factors including: 1) increased presentation rate of ACTH to adrenocortical cells, 2) release of endothelin-1 from the endothelium in response to shear stress, which has been shown to directly stimulate steroid secretion from adrenocortical cells (reviewed by Bassett & West, 1997).

The finding that changes in blood flow can modulate steroid secretion irrespective of the presence of ACTH implies an indirect function for vasodilators in the regulation of steroidogenesis. ACTH itself causes vasodilation by inducing the release of the vasodilators, serotonin (5HT) and histamine, from mast cells located in the capsular region near arterioles. Stimulation of the splanchnic nerve results in increased blood flow and perfusion rate through the adrenal, probably resulting from the release of vasoactive neuropeptides such as VIP. VIP has also been shown to increase perfusion medium flow rate through the rat adrenal with a concomitant increase in corticosterone production (Hinson *et al*, 1994a).

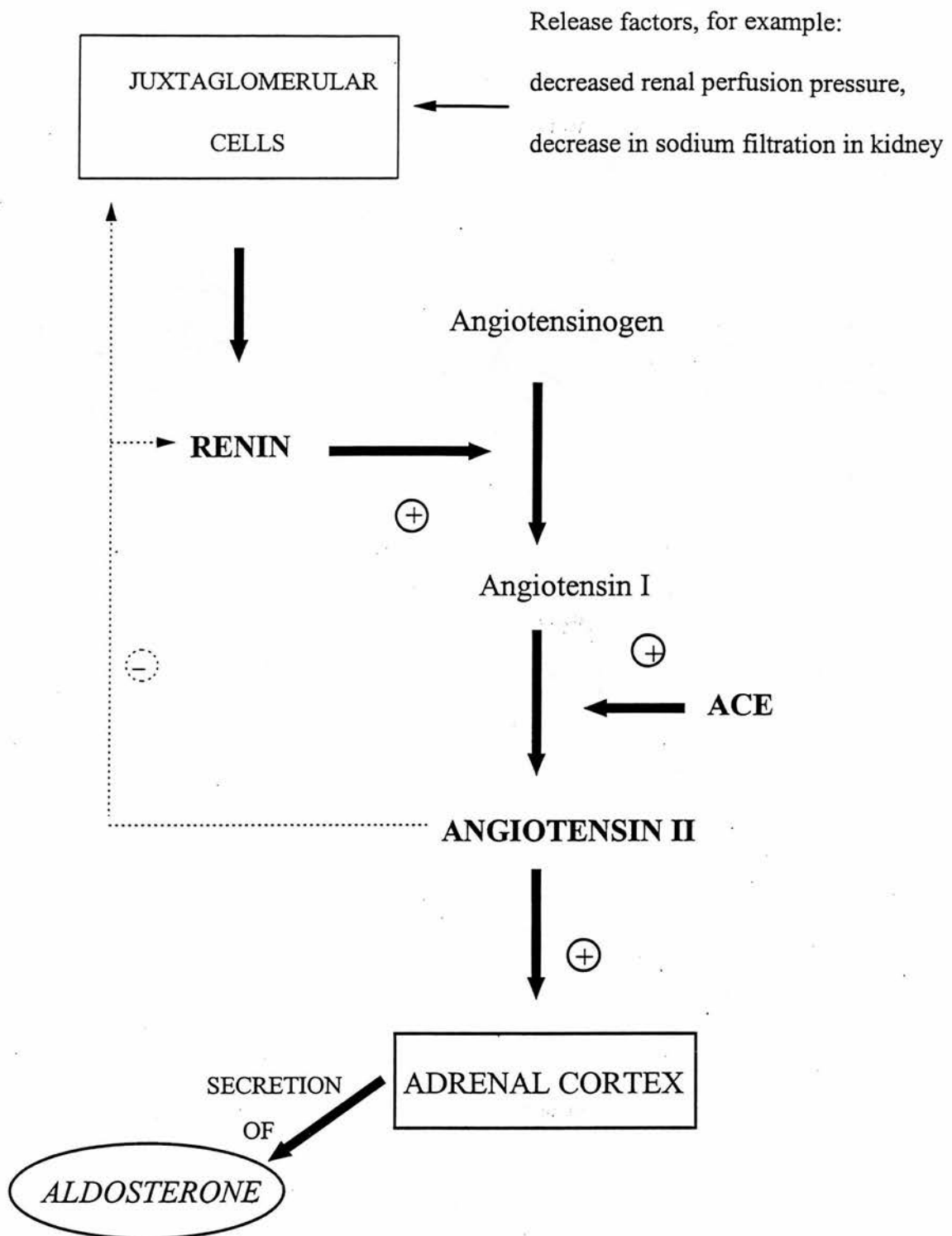


Figure 1.5

Simplified illustration of the pathway of the RAS leading to stimulation of aldosterone secretion from the adrenal cortex.

There is evidence that adrenal blood flow may also be regulated by modulation of venous outflow from the gland (reviewed by Bassett & West, 1997). The concept of vascular damming was first raised by Dobbie & Symington in 1966. They postulated that blood could be trapped within the adrenal gland at the site of the cortico-medullary junction, by a damming effect created by the transition from the vessel-abundant vascular plexus in the zr to the relatively few vessels within the medulla and the presence of longitudinal muscle bands within the thin-walled venous radicles which empty into the central vein. It has been suggested that the entrapment of blood within the adrenal may provide a site for storage of glucocorticoid which can then be released quickly into the circulation in response to stress. Rapid release of venous congestion is probably mediated by a vasodilator released locally within the medulla. Intra-adrenal CRH has been suggested to be a likely candidate based on the following: 1) CRH is a vasodilator, 2) CRH has been detected in the medulla, 3) levels of CRH within the venous outflow increase following stimulation of the splanchnic nerve 4) glucocorticoid secretion is increased in the presence of CRH if the gland architecture is intact.

1.6.4 Intra-adrenal regulation of steroidogenesis

The previous section dealt with 'traditional' control mechanisms for steroidogenesis. Accumulating evidence, however, exists for additional regulatory mechanisms. These included observations that increased glucocorticoid secretion did not always correlate with projected ACTH concentration or presentation rate in the adrenal (see Charlton, 1990, for review). For example, an increase in glucocorticoid secretion is detected in cases of raised intracranial pressure following acute brain injury in man which is not accompanied by a corresponding rise in plasma ACTH concentration (Feibal *et al*, 1983). Engeland *et al* (1981, 1985) demonstrated ACTH-independent changes in adrenocortical sensitivity and increased cortisol secretion in response to small haemorrhage in awake dogs that appeared to be independent of ACTH presentation rate. Also, corticosteroid secretion rhythms can be dissociated from ACTH rhythms in rats (Dallman *et al*, 1978) and dogs (Engeland *et al*, 1981).

Several putative neurotransmitters, such as VIP, have been detected in the adrenal cortex and, together with the accumulating morphological evidence for adrenocortical innervation (section 1.6.4.1), has indicated a neural control mechanism in the adrenal cortex. In addition to a postulated direct neural regulation (via nerve endings in the cortex in close contact with cortical cells), indirect mechanisms involving neural regulation of a) adrenocortical blood flow, b) cell growth or c) release of medullary peptides (with a putative paracrine action; section 1.6.4.2) may also affect adrenocortical cell function.

1.6.4.1 Adrenocortical Innervation

The adrenal medulla has a well documented supply of preganglionic sympathetic, cholinergic, nerve fibres, arising from the splanchnic nerve and terminating directly on the medullary chromaffin cells (Symington, 1969). These nerves are involved in the secretion of medullary catecholamines.

In 1894 Dogiel described a rich nerve plexus within the adrenal cortex of mammals, including humans. Despite this, the question of adrenocortical innervation remained controversial until relatively recently. A series of studies by Dallman *et al* (1976) found that nerves were responsible for the compensatory adrenal growth following unilateral adrenalectomy, providing evidence for a direct effect of innervation on the adrenal cortex. Splanchnic innervation also modulates adrenal blood flow, indirectly influencing steroidogenesis (Engeland and Gann, 1989; Edwards *et al*, 1986) (section 1.6.3).

Anatomical studies, involving the use of histological, electron microscopic and catecholamine fluorescence techniques, demonstrated the presence of autonomic axons within the adrenal cortex in the rat, pig (Unsicker, 1971), guinea-pig and human (Garcia-Alvarez, 1970, Mikhail & Amin, 1969). Some of these fibres were in close contact with cortical cells, implying a direct influence of nerves on steroidogenesis.

A neuronal system dependent on splanchnic nerve activity has been described in the rat, with cell bodies arising in the adrenal medulla and fibres running between the medulla and the cortex (Holzwarth *et al*, 1987). In addition, two postganglionic sympathetic nerve plexuses (the afferent (sensory) and the efferent systems) are present; both run alongside arteries, have their cell bodies outside the adrenal and appear to be independent of splanchnic nerve stimulation (Holzwarth *et al*, 1987).

More recently, immunohistochemical staining of a neurone-specific marker, ubiquitin hydrolase protein gene product 9.5 (PGP 9.5), demonstrated a number of morphologically different nerves in the human adrenal cortex (McNicol *et al*, 1994). Although large nerve trunks were seen traversing the cortex to the medulla, without apparent synapsing in the cortex, networks of slender nerve bundles and individual fibres were found in all adrenocortical zones. These fibres exhibited varicosities indicative of neurotransmitter release. This supported the findings of Dorovini-Zis & Zis (1991), who reported the presence of nerve terminals in close contact with zona fasciculata endocrine cells in man. A variety of putative neurotransmitters have been detected in association with adrenocortical nerves (reviewed by Vinson *et al*, 1994), a few of which are briefly discussed below. Some of these neurotransmitters were found co-localised within the same nerve fibres.

(a) Cholinergic innervation

The presence of acetylcholinesterase (AChE)-positive nerve fibres and cell bodies within the ovine adrenal cortex was described by Robinson *et al* (1977). These fibres, possibly derived from the splanchnic nerve, were distributed within the cortical parenchymal tissue and formed a distinct neural plexus in the zr. In the human adrenal, radial trunks of AChE-positive nerves traversed the zona fasciculata from a subcapsular plexus, often linking with a second neural plexus in the zr (Charlton *et al*, 1991; Gilchrist *et al*, 1993). Individual nerve fibres, branching from these nerve trunks, synapsed within the cortical parenchyma and appeared to follow the distribution of the capillaries in the cortex, consistent with a nerve supply to the cortical cells and/or blood supply (Charlton *et al*, 1991). Indeed, acetylcholine

modulated cortisol secretion directly and also via changes in blood flow in calves (Edwards & Jones, 1993a, 1993b). Acetylcholine has also been found to directly stimulate steroidogenesis from isolated, perfused calf adrenals (Rosenfeld, 1955), bovine zfr cells (Hadjian *et al*, 1982), bovine zg cells (Kojima *et al*, 1986), frog interrenal tissue (Benyamina *et al*, 1987) and rat adrenal glands (Porter *et al*, 1988). A muscarinic receptor was postulated to be involved and the presence of this receptor subtype within the bovine adrenal cortex reported (Hadjian *et al*, 1981). The mechanism of action of ACh involved a stimulation of phosphatidylinositol turnover (Hadjian *et al*, 1984; Kojima *et al*, 1986; Walker *et al*, 1990) and increased levels of intracellular Ca^{2+} (Kojima *et al*, 1986).

(b) Catecholaminergic Innervation

This area has been reviewed in detail by Tóth *et al* (1997). In brief summary, catecholamine fluorescence studies and immunohistochemical detection of dopamine β -hydroxylase (DBH; an enzyme involved in noradrenaline synthesis) have revealed the presence of catecholaminergic neurones in the sheep (Robinson *et al*, 1977), rat (Holzwarth *et al*, 1987) and human adrenal cortex (Charlton *et al*, 1992). The nerves were principally noradrenergic (as indicated by the absence of phenylethanolamine N-methyltransferase (PNMT; the enzyme which converts noradrenaline to adrenaline) immunoreactivity (Holzwarth *et al*, 1987; Oomori *et al*, 1994)) and located mainly in the subcapsular and zona glomerulosa regions. The distribution, in the human, was similar to the cholinergic innervation, with a second plexus detected within the outer zr. The nerves did not appear to penetrate the inner zr or the medulla (Charlton *et al*, 1992). Nerve fibres were detected in the muscular walls of cortical blood vessels (Charlton *et al*, 1992), consistent with a modulatory effect of postganglionic adrenergic nerves on adrenal blood flow (Carlsson *et al*, 1993). Branching fibres were also found in close contact with endocrine cells (Charlton *et al*, 1992), supporting a direct steroidogenic action for catecholamines. The catecholamine content of many adrenergic neurones found in the zg region was unaffected by splanchnic nerve input and disappeared following chemical

sympathectomy (Holzwarth *et al*, 1987), indicating an extra-adrenal origin of some noradrenergic nerves in the adrenal.

Catecholamines have been shown to stimulate increased aldosterone secretion from zg cell preparations of human adrenals (Neri *et al*, 1996). In addition, catecholamines regulated steroidogenesis from other mammalian inner zone cells; Lightly *et al* (1990) reported increased glucocorticoid secretion from bovine zfr cells and Ehrhart-Bornstein *et al* (1994) demonstrated androstenedione secretion from porcine zfr preparations. Adrenaline stimulated androstenedione release from isolated, perfused pig adrenals which was antagonised by the β -adrenergic antagonist, propranolol, but not by the α -adrenergic antagonist, phentolamine (Ehrhart-Bornstein *et al*, 1994). Catecholamine-induced steroidogenesis was accompanied by up-regulation of steroidogenic enzyme transcripts (Ehrhart-Bornstein *et al*, 1991b).

(c) VIPergic Innervation

The presence of VIPergic innervation in the adrenal gland has been studied most extensively in the rat (Hökfelt *et al*, 1981; Holzwarth *et al*, 1987; Oomori *et al*, 1994) but has also been shown to exist in the pig (Kong *et al*, 1989). Immunohistochemical studies revealed the presence of a predominant VIPergic nerve plexus in the capsule-zg region, often associated with blood vessels (Hökfelt *et al*, 1981; Holzwarth *et al*, 1987). However, some fibres were seen extending into the deeper layers of the cortex (Holzwarth *et al*, 1984) and running between the medulla and the cortex (Hökfelt *et al*, 1981) and appeared to innervate clusters of cortical cells (Holzwarth *et al*, 1987).

Some VIPergic nerves probably constitute part of the intrinsic neural network connecting the medulla and cortex, which is dependent on splanchnic nerve activity. Some intra-adrenal ganglion cells were VIP-immunoreactive (Hökfelt *et al*, 1981; Oomori *et al*, 1994). Splanchnic nerve ligation increased VIP-immunostaining in the adrenal (Holzwarth *et al*, 1987) whilst stimulation of the splanchnic nerve resulted in VIP release from the gland, detected in the adrenal vein of conscious calves (Bloom

et al, 1987) and from isolated, perfused porcine and rat adrenals (Ehrhart-Bornstein *et al*, 1991a; Wakade *et al*, 1991). In addition, Hökfelt (1981) reported that extrinsic denervation of the adrenal gland did not affect the presence of VIP-immunoreactive fibres and ganglion cells.

It is possible that VIPergic nerves may arise from both intra- and extra-adrenal ganglia. Oomori *et al* (1994) reported that VIP-containing nerves and ganglion cells in the medulla were non-catecholaminergic and immunonegative for neuropeptide Y (NPY), different to those found in the superficial cortex (which were often immunopositive for NPY, dopamine β -hydroxylase (DBH) and tyrosine hydroxylase (TH)). The VIP-, NPY-, DBH- and TH-immunopositive fibres were mainly distributed in the zg and often associated with blood vessels. Hinson & Kapas (1996) reported that splanchnic nerve section affected VIP immunoreactivity in the medulla and inner zones of the cortex but not in the capsule-zg. This supports a dual origin for VIPergic nerves in the adrenal and suggests that VIP may regulate outer and inner cortical function in different ways.

(d) Other peptidergic innervation

These include NPY, calcitonin gene-related peptide (CGRP) and corticotrophin releasing factor (CRF).

NPY-immunoreactive nerve fibres have been detected in the adrenal cortex of several mammalian species including horse, rat, mouse and cow (Varndell *et al*, 1984; Majane *et al*, 1985). In the rat, a network of NPY-immunopositive nerves was identified in the subcapsular-zg region, associated mainly with blood vessels (Kuramoto *et al*, 1986). NPY was sometimes found co-localized with VIP in nerve fibres in the rat outer adrenal cortex (Oomori *et al*, 1994). Receptors for NPY have been detected using autoradiography in the bovine zg but not in other regions of the gland (Torda *et al*, 1988). Direct modulation of steroidogenesis by NPY is controversial. Although NPY-stimulated aldosterone secretion from adrenal slices

was blocked by NPY receptor antagonists (Mazzocchi *et al*, 1996a), Hinson & Kapas (1995) found no secretagogue effect of NPY on dispersed rat zg cells. Similar conflicting data exists for dispersed rat inner cortical cells; Hinson *et al* (1994b) noted a small increase in corticosterone secretion from *in situ* perfused rat adrenals but not from dispersed rat zfr cells (Hinson *et al*, 1995). It has been postulated that the steroidogenic action of NPY may be indirect, either through modulation of blood flow (NPY-induced secretion of corticosterone from *in situ* rat perfused adrenals was accompanied by a change in perfusion medium flow rate (Hinson *et al*, 1994a) or via NPY-stimulated release of catecholamines (NPY elicits catecholamine release by chromaffin cells and the β_1 -adrenoceptor antagonist, atenolol, inhibited NPY-mediated aldosterone release by rat capsule-zg preparations (Bernet *et al*, 1994a, 1994b).

Varicose CGRP-containing nerves have been detected in the capsule-zg region of the rat adrenal cortex, in close apposition to zg cortical cells (Kuramoto *et al*, 1987). Most fibres, however, traversed the cortex to synapse within the medulla. CGRP-positive fibres have also been found in the adrenal cortex of guinea-pigs (Heym *et al*, 1995) and pigs (Kong *et al*, 1989). The steroidogenic effect of CGRP appears conflicting. Although CGRP increased aldosterone and corticosterone secretion from isolated perfused rat adrenals, accompanied by an increase in perfusion medium flow rate (Hinson & Vinson, 1990), administration to rats decreased plasma aldosterone levels (Mazzocchi *et al*, 1992) and inhibited AII-stimulated aldosterone secretion by rat zg cells (Mazzocchi *et al*, 1996b). Bloom *et al* (1989) reported an increase in cortisol secretion, from functionally hypophysectomised calves, which could not be explained by increased blood flow and ACTH presentation rate alone.

CRH-immunoreactive nerve fibres have been detected in the adrenal cortex of sheep and rat zg (Rundle *et al*, 1988). Steroidogenic actions of CRH may be indirect. CRH failed to elicit a steroid response from dispersed zg cells (Neri *et al*, 1991) and was shown to increase adrenal blood flow in calves (Jones & Edwards, 1992). Along with effects on adrenal blood flow, CRH has been reported to increase adrenal

sensitivity to ACTH in rats (van Oers *et al*, 1992). Administration of a CRH antagonist to conscious calves, however, indicated that CRH does not mediate the response to splanchnic nerve stimulation (Jones & Edwards, 1991).

1.6.4.2 Cortico-medullary Interactions

The adrenal medulla and the adrenal cortex have long been considered to be two anatomically and functionally distinct endocrine tissues. Together with the presence of a neural network linking the medulla to the cortex (section 1.6.4.1), further evidence has recently been presented which supports both a morphological and functional interaction between these two glands greater than hitherto suspected.

(a) Cortical actions on the medulla

Adrenocortical steroid hormones influence the functioning of the medulla (reviewed by Axelrod and Reisine, 1984). Glucocorticoids regulate the synthesis of adrenaline by modulating the activity of the medullary enzyme PNMT (an enzyme involved in the conversion of noradrenaline to adrenaline, see *Figure.1.6*) as well as exerting a (lesser) control of the noradrenaline-forming enzyme, DBH. Presentation of corticosteroids to medullary chromaffin cells is aided by the centripetal blood flow from cortex to medulla in the adrenal. In addition, adrenocortical cells, immunopositive for the steroidogenic enzyme 17 α -hydroxylase, have been found scattered throughout the medulla of porcine, bovine and human adrenals (Bornstein *et al*, 1991, 1994).

(b) Medullary action on the cortex

This area has been extensively reviewed by Nussdorfer (1996). The medulla is innervated by preganglionic, cholinergic, sympathetic neurones carried in the splanchnic nerves. Stimulation of the splanchnic nerve results in the release of

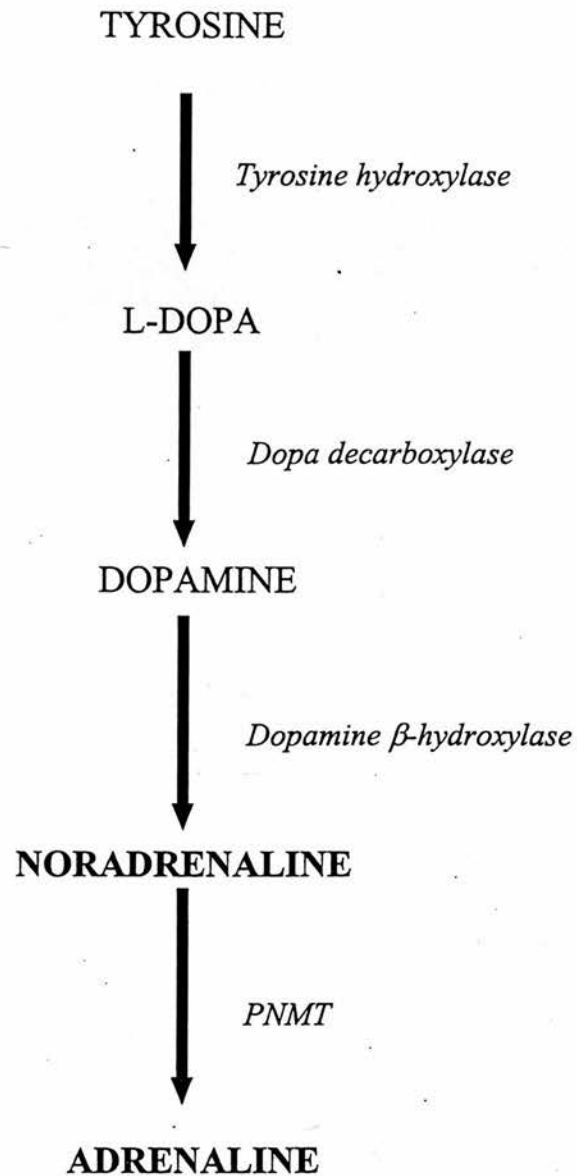


Figure 1.6

Illustration of the pathway for catecholamine synthesis in medullary chromaffin cells. Some chromaffin cells do not contain PNMT and therefore cannot synthesise adrenaline. PNMT = phenylethanolamine-N-methyl transferase.

chromaffin cell products, principally catecholamines. Over recent years, evidence has emerged that the medulla may influence cortical function via a splanchnic nerve-dependent mechanism (reviewed by Charlton, 1990; Edwards and Jones, 1993b). Neurotransmitters released from splanchnic nerves may stimulate adrenocortical cells 1) directly, from free nerve endings within the cortex or 2) indirectly, by stimulating chromaffin cells present within the medulla/adrenal cortex, causing the release of chromaffin cell secretagogues. These, in turn, could modulate adrenocortical function in a paracrine manner via specific receptors on the adrenocortical cells. Although these chromaffin cell secretagogues may affect steroidogenesis directly, they may act indirectly as well/instead, for example, by modulating adrenal blood flow or activation of an intra-adrenal RAS. Also, sectioning of the splanchnic nerves has been shown to decrease ACTH-induced glucocorticoid secretion from conscious, functionally hypophysectomised calves (Edwards & Jones, 1987) and stimulation of the splanchnic nerves to increase androstenedione secretion from isolated, perfused pig adrenals (Ehrhart-Bornstein, 1994).

The predominant agents released from chromaffin cells following splanchnic nerve activation are the catecholamines, adrenaline and noradrenaline. Both have been shown to directly affect steroid secretion from outer and inner zone adrenocortical cells, via adenylate cyclase-coupled β -adrenoceptors (see review by Nussdorfer *et al*, 1996, and section 1.6.4.1b).

Other steroidogenic peptides have been detected in the adrenal medulla. It has recently been demonstrated that human medullary chromaffin cells secrete VP (Guillon *et al*, 1995) and that VP can stimulate both aldosterone and cortisol secretion from human adrenocortical cells (Guillon *et al*, 1995; Perraudin *et al*, 1993).

ANP mRNA has been detected in the adrenal medulla of humans (Lee *et al*, 1994) and rats (Morel *et al*, 1988). ANP inhibited both basal and AII-stimulated aldosterone release from mammalian zg cells (reviewed by Nawata *et al*, 1991).

Chromaffin granules release high concentrations of ATP (Rojas *et al*, 1986)

which is known to stimulate cortisol release from bovine inner zone adrenocortical cells (Hoey *et al*, 1994).

Further indirect evidence for a paracrine regulation of the cortex by medullary peptides/amines relates to the identification of chromaffin tissue within the adrenal cortex. By using immunostaining techniques for the specific neuroendocrine markers, synaptophysin and chromogranin A (as markers for chromaffin cells), rays of chromaffin tissue, extending from the medulla, along with small clusters and single chromaffin cells, have been observed in all zones of rat, porcine and human (Bornstein *et al*, 1991, 1994) adrenal cortex. These chromaffin cells are in close contact with adrenocortical cells, with no separating interstitium (Bornstein *et al*, 1991, 1994). Ultrastructural evidence showing exocytosis from a chromaffin cell in direct apposition to an adrenocortical cell has been documented and lends further support to a paracrine regulation of steroidogenesis by chromaffin cell products (Bornstein *et al*, 1992).

1.6.5 The intra-adrenal RAS

All the components of the systemic RAS, namely, renin, angiotensinogen, ACE, AI and AII, have been identified in the rat adrenal cortex, indicating the presence of an adrenal RAS (reviewed by Mulrow, 1998). The adrenal RAS has been further localised primarily to the zg, with greater than 90 % of renin activity found in zg cells (Doi *et al*, 1984). The majority of extractable AI and AII was also detected in zg cells (Nakamaru *et al*, 1985). Most studies on the intra-adrenal RAS have been performed on the rat but the presence of renin in the human adrenal has been observed (Naruse *et al*, 1983).

Although no definite physiological role for this local system has been identified as yet, evidence exists to support a function in the regulation of aldosterone (reviewed by Mulrow, 1998). In rats, adrenal renin activity and expression increased following sodium depletion, accompanied by increased aldosterone production (Doi *et al*, 1984). In addition, a high potassium diet caused an increase in adrenal renin and aldosterone secretion whilst plasma renin activity decreased (Doi *et al*, 1984).

Inhibition of the RAS, using ACE inhibitors, in superfused normal human adrenal tissue and cultured bovine zG cells (therefore unexposed to stimulation by the systemic RAS) showed a decrease in both AII production and aldosterone secretion (Fallo *et al*, 1991; Horiba *et al*, 1990).

The precise mechanism of action of locally produced AII on steroidogenesis is still unclear. Possibilities include an autocrine mechanism whereby AII binds to intracellular (perhaps nuclear) AII receptors, or a paracrine action whereby AII released from one cell activates cell-surface receptors on adjacent cells (Mulrow, 1998).

1.6.6 The intra-adrenal CRH-ACTH system

CRH and ACTH immunoreactivity (Suda *et al*, 1986) have been detected in the human adrenal medulla and CRH stimulated the release of ACTH from adrenal fragments (Andreis *et al*, 1992), suggesting the presence of an intra-adrenal CRH-ACTH system which may locally regulate adrenal function. CRH-containing cells and nerves have also been detected in ovine adrenal (Rundle *et al*, 1988).

A direct action of CRH on adrenocortical function appears unlikely. Adenylate cyclase-coupled CRH receptors have been detected in the monkey adrenal medulla which, when activated, stimulate catecholamine secretion (Udelsman *et al*, 1985). In conjunction with a stimulatory action on intra-adrenal ACTH release (Andreis *et al*, 1992), this suggests an indirect modulation of steroidogenesis by CRH. Udelsman *et al* (1985) failed to detect the presence of CRH receptors on adrenocortical cells. Locally secreted ACTH, however, probably acts in a direct paracrine fashion to stimulate steroid secretion from adrenocortical cells. The physiological significance of an intra-adrenal CRH-ACTH system is still unclear though disturbance of the systemic CRH-ACTH system, for example, by hypophysectomy, results in an increase in adrenal CRH and ACTH immunoreactivity (Mazzocchi *et al*, 1994a).

1.7 Vasoactive Intestinal Peptide (VIP)

VIP is a 28 amino acid peptide (see *Figure 1.7*) and is a member of a family of peptides which includes pituitary adenylate cyclase activating polypeptide (PACAP) and growth hormone releasing hormone (GHRH).

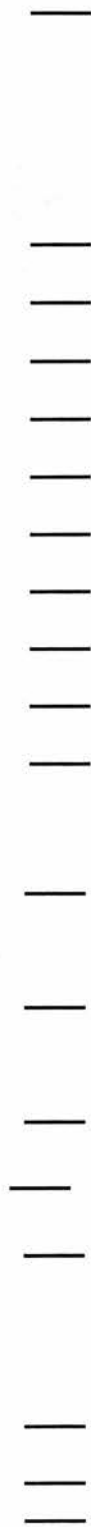
The human VIP gene has been isolated (Itoh *et al*, 1983; Okamoto *et al*, 1983). It contains seven exons (Gozes *et al*, 1986) and encodes at least two peptides; exon 5 codes for VIP and exon 4 encodes peptide-histidine-methionine (PHM) in humans (peptide-histidine-isoleucine (PHI) in the rat) (Nishizawa *et al*, 1985). Another peptide, PHV (peptide-histidine-valine), can also be produced via a different cleavage site between PHM and VIP and has been associated with smooth muscle relaxation (Spokes *et al*, 1988).

The physiological importance of VIP is indicated by the high degree of homology between VIP mRNA in humans and rats (80-90 %), with 90% homology in the VIP-coding domain (Nishizawa *et al*, 1985). VIP was first isolated from porcine gut (Said & Mutt, 1970), where it was found to have vasoactive and secretory properties. Since then, VIP has been detected in the central and peripheral nervous systems and in neuroendocrine cells, where it exhibits neurotransmitter and hormonal actions. The actions attributed to VIP are varied; for example, VIP exerts effects in the gastrointestinal system, including stimulation of gastric motility (Reid *et al*, 1988), in the central nervous system and brain, including cerebral vasodilation (Lee *et al*, 1984) and memory formation (Gozes *et al*, 1993) and in the reproductive system, including oocyte maturation in the ovaries (Tornell *et al*, 1987), amongst others. In particular, VIP acts as a secretagogue in a number of tissues, for example, it stimulates secretion of prolactin from the anterior pituitary (Ehjalbert *et al*, 1980), thyroid hormone (Ahren & Hedner, 1989) and catecholamines from the canine adrenal medulla (Yamaguchi, 1993).

VIP has been localised, predominantly in neurons, in a wide number of tissues. In the brain, it has been detected in high levels in the cerebral cortex (Said, 1986), hypothalamus (particularly the suprachiasmatic nucleus) (Baldino *et al*, 1988; Said, 1986) and anterior pituitary (Morel *et al*, 1981) as well as other regions of the

VIP

H₂N-His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-CONH₂



H₂N-His-Ser-Asp-Gly-Ile-Phe-Thr-Asp-Ser-Tyr-Ser-Arg-Tyr-Arg-Lys-Lys-Tyr-Leu-Ala-Val-Lys-Lys-Tyr-Leu-Ala-Val-Leu-CONH₂

PACAP27

PACAP38

H₂N-His-Ser-Asp-Gly-Ile-Phe-Thr-Asp-Ser-Tyr-Ser-Arg-Tyr-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Ala-Val-Leu-Gls-Lys-Arg-

Tyr-Lys-Gln-Arg-Val-Lys-Asn-Lys-CONH₂

Figure 1.7

Amino acid sequence of VIP and the related peptides, PACAP27 and PACAP38. The sequence identity between PACAP27 and VIP is indicated (solid lines). The VIP amino acid sequence is identical in humans, rats and pigs.

brain and central nervous system. VIP is often found co-localised with acetylcholine in nerve endings (Hokfelt *et al*, 1980).

VIP has been detected in nerve terminals associated with the gastrointestinal tract (Wattchow *et al*, 1988) and the tracheobronchial tree (Dey *et al*, 1988) and has been detected in placenta and mast cells (Said, 1991, for review). Of particular importance to this study was the detection of VIP and its transcript in the adrenal medulla, within nerve terminals in the medulla and cortex and ganglion cells and chromaffin tissue in the medulla (Hokfelt *et al*, 1981; Holzwarth *et al*, 1987; Oomori *et al*, 1994). Evidence for a function of VIP in the regulation of adrenal steroidogenesis is described in section 4.1.1.

1.8 The H295R human adrenocortical carcinoma cell-line

The NCI-H295 cell-line was initially characterised by Gazdar *et al* (1990) 7-10 years after isolation of the cells from a primary adrenocortical carcinoma removed from a female patient exhibiting signs of glucocorticoid, mineralocorticoid and androgen excess. The cell-line was originally established as a population of cells which grew in suspension, due to difficulties associated with fibroblast growth (Gazdar *et al*, 1990). Unstimulated cells were reported to secrete more than 30 steroids, with the identification of approximately 20 of these (Gazdar *et al*, 1990).

More recently, a subpopulation of the original cells has been established which is capable of growing as a monolayer culture (Rainey *et al*, 1993; 1994). This population of cells, available from the ATCC and often referred to as H295R cells, has recently been characterised (Rainey *et al*, 1994, review). The cells have been found to express the major steroidogenic enzymes required for synthesis of steroids from the mineralocorticoid, glucocorticoid and adrenal androgen pathways, including P450_{scc}, P450_{c17}, P450_{c21}, P450_{c18} and 3 β HSD (Staels *et al*, 1993) and have been shown to be capable of secreting the principal end-products of these pathways; aldosterone, cortisol and androstenedione (Bird *et al*, 1993; Rainey *et al*, 1993; 1994 (review)). Moreover, studies have shown that the H295R cell-line is

steroidogenically responsive to a number of physiological agonists, including AII (Bird *et al*, 1993), K⁺ (Bird *et al*, 1995a) and, to a lesser extent, ACTH (Rainey *et al*, 1993). Certain key steroidogenic enzymes also appear to be regulated differently by AII and the protein kinase A activator, forskolin (Rainey *et al*, 1994). In particular, forskolin increased expression of P450c17 mRNA most potently, with lesser effects on P450scc and 3 β HSD transcripts. In contrast, AII increased 3 β HSD mRNA to the greatest extent, with lesser effects on P450scc and P450c17 transcripts (Mason *et al*, 1995; Rainey *et al*, 1994).

1.9 Aims of the thesis

- 1) To investigate the short-term and long-term effects of AII and forskolin on the secretion of cortisol, corticosterone, aldosterone and androstenedione from the H295R human adrenocortical carcinoma cell-line.
- 2) To investigate whether the steroid phenotype of the cell-line could be altered to a relatively zg-like cell or a relatively zfr-like cell by pretreatment of H295R cells with AII or forskolin and measurement of the subsequent steroid responses to 4 h AII treatment.
- 3) To investigate a direct action of VIP on steroid secretion from H295R cells and to characterise this response in terms of second messenger systems activated and receptor types involved, using specific receptor agonists and antagonists, including VPAC receptor subtype-selective agonists and antagonists, and RT-PCR analysis of VPAC1, VPAC2 and PAC1 receptor transcripts.
- 4) To undertake a preliminary investigation of the effects of a range of putative paracrine and neuroendocrine agonists (ANP, ATP, 5-HT, VP, ACh, NA) on steroid secretion from H295R cells.

2.0 MATERIALS AND METHODS

2.1 MATERIALS

All chemicals were AnalaR grade and except where otherwise stated were obtained from BDH Merck or Sigma Chemical Company.

2.1.1 Cell-line culture materials

American Type Culture Collection, Maryland, USA

NCI-H295R human adrenocortical carcinoma cell-line

Gibco BRL (Life Technologies) Ltd., Renfrew

Dulbecco's modified Eagle's medium / Ham's F12 nutrient mix (DMEM / F12), Earle's Balanced Salt solution (EBSS), penicillin / streptomycin, trypsin (0.25%) / EDTA (0.2%) solution, Ultrosor serum replacement

Mackay & Lynn Ltd., Edinburgh

Corning tissue culture plasticware (75 cm² flasks, 12-well plates)

Sigma Chemical Co. Ltd., Poole, Dorset

ITS (0.5 µg/ml insulin, 0.5 µg/ml transferrin, 0.5 ng/ml sodium selenite)

2.1.2 Agonists and antagonists

Boehringer Mannheim UK, Lewes, E. Sussex

adenosine 5' triphosphate (ATP)

Ciba Laboratories, Horsham, W. Sussex

ACTH (1-24; synacthen)

Martindale Pharmaceuticals, Romford, Essex

adrenaline

NIBSC, Potters Bar, Herts.

angiotensin II (asp¹-val⁵; WHO standard 64/15), human atrial natriuretic factor (ANP 99-126; WHO standard 85/669)

Novabiochem, Switzerland

pituitary adenylate cyclase activating polypeptide 1-38 (PACAP 38)

Parke-Davis, Pontypool, Gwent

arginine vasopressin (pitressin)

Pharmax Ltd., Bexley, Kent

isoprenaline (hydrochloride; as Saventrine)

Sanofi Winthrop Ltd., Guildford, Surrey

noradrenaline (tartrate; as levophed)

Sigma Chemical Co. Ltd., Poole, Dorset

acetylcholine chloride (ACh), human atrial natriuretic peptide (ANP), vasoactive intestinal peptide (VIP; porcine, synthetic), [D-p-Cl-Phe⁶, Leu¹⁷]-VIP (human, porcine, rat; synthetic)

Zeneca Ltd., Macclesfield, Cheshire

propranolol (Inderal)

2.1.3 Radioimmunoassay materials

Amersham International Plc, Little Chalfont, Bucks

cortisol tracer (cortisol -3-(O-carboxymethyl) oximino-(2-[¹²⁵I] iodohistamine)), specific activity 2000 Ci / mmol.

Scottish Antibody Production Unit (SAPU), Carlisle, Lanarkshire

antisera to cortisol (batch no.: 7250X), non-immune sheep serum (batch no.: 7414Y), donkey anti-sheep (goat) IgG (batch no.: 7426Y), donkey anti-rabbit IgG (batch no.: 7144X), normal rabbit serum (batch no.: 7374Y).

Sigma Chemical Co. Ltd., Poole, Dorset

aldosterone, androstenedione, corticosterone, charcoal, dextran, cortisol, sodium azide

Dr. B.C. Williams, Department of Medicine, Western General Hospital, Edinburgh, Scotland kindly supplied:

cAMP tracer (adenosine 3',5'-cyclic phosphoric acid 2'-O-succinyl-3 [¹²⁵I] iodotyrosine), cGMP tracer (guanosine 3',5'-cyclic phosphoric acid 2'-O-succinyl-3 [¹²⁵I] iodotyrosine), cAMP antibody, cGMP antibody, aldosterone tracer (aldosterone-3(O-carboxymethyl)oximo-(2[¹²⁵I]iodohistamine), aldosterone polyclonal antibody, corticosterone tracer (corticosterone-3(O-carboxymethyl)oximo-(2[¹²⁵I]iodohistamine), corticosterone polyclonal antibody, cAMP stock standard, cGMP stock standard.

Dr. M. Wallace, Dept. Clinical Chemistry, Royal Infirmary, Glasgow, Scotland

kindly donated: androstenedione tracer (androstenedione-3-carboxymethyloxime [¹²⁵I]iodohistamine), androstenedione antibody

2.1.4 Molecular biology materials

Stratagene Ltd., Cambridge

RT-PCR kit

Dr. E. Lutz, MRC Brain Metabolism Unit, Royal Edinburgh Hospital, Scotland

kindly donated: VPAC1 receptor primers, VPAC2 receptor primers, PACI receptor primers, all VPAC1 receptor, VPAC2 receptor and PACI receptor positive control material for PCR reactions, HPRT primers

2.1.5 Phosphoinositols assay and protein assay materials

Aldrich Chemical Co. Ltd., Gillingham, Dorset

1,1,2-trichlorotrifluoroethane, tri-n-octylamine

Amersham International Plc, Little Chalfont, Bucks

myo-[³H]-inositol, specific activity 81 Ci / mmol.

BDH Merck Ltd., Lutterworth, Leicestershire

formic acid (ammonium salt), lithium chloride, perchloric acid, triethylamine, ethylenediamine tetraacetic acid (EDTA, disodium salt)

Bio Rad Laboratories Ltd., Hemel Hempstead, Herts.

AG1-X8 anion exchange resin

Canberra Packard, Pangbourne, Berks

scintillation vials, liquid scintillant (Ultima Gold)

Sigma Chemical Co. Ltd., Poole, Dorset

ammonium formate, bovine serum albumin (BSA) (cell culture grade, fraction V),
laurylsulphate (sodium salt; SDS)

2.2 METHODS

2.2.1 NCI-H295R Cell-Line Culture

NCI-H295R (H295R) cells were grown in 75 cm² tissue culture flasks and maintained in growth medium (DMEM / F12 (1:1) (10 ml per flask) containing 2% Ultrosor and 1% ITS solution) at 37°C under an atmosphere of 95% air/5% CO₂. Exhausted medium was replaced with fresh medium every 2-3 days.

Cells were subcultured upon reaching confluence using trypsin (0.25%) / EDTA (0.2%) solution, 5 ml / 75 cm² flask for 60 secs. This was usually a period of 7-10 days, due to the low proliferation rate of these cells. At monthly intervals cells were routinely subcultured into growth medium containing penicillin (50 IU/ml) and streptomycin (50 µg/ml) and maintained in this medium prior to further passage. This was to remove any low-level bacterial infections which may have been present. Every three to four months, cells were also tested for the presence of any mycoplasma infection. This was performed by fixing the cells in acetone and then staining DNA (cell nuclei and mycoplasma, found in cytosol) using Hoechst 33258 fluorochrome, followed by visualisation using a UV spectrophotometer.

2.2.2 Treatment of cells with agonists and antagonists

2.2.2.1 Chronic treatment of H295R cells.

H295R cells were seeded into 12-well plates and maintained as described in 2.2.1 until confluent (7-10 days). Cells were then incubated with fresh growth medium (as defined in 2.2.1) with or without AII (10 nM) or forskolin (10 µM) for a period of 96 h, this medium being replaced with fresh agonist-containing medium every 24 h. Medium from each 24 h period was collected, stored at -20°C, and subsequently analysed for cortisol, corticosterone, androstenedione and aldosterone content by radioimmunoassay (RIA) procedures. H295R cells were also treated in the same manner with VIP (concentrations as stated in chapter 4.0) for up to 96 h and

with a range of putative agonists (ANP, ATP, 5-HT, VP, ACh, NA; concentrations as stated in chapter 7.0) for 24 h.

2.2.2.2 Short-term stimulation of H295R cells following chronic treatment.

Following the chronic treatment phase with AII or forskolin, as detailed in 2.2.2.1, cells were incubated with or without AII (10 nM) for 4 h in order to determine any possible effect of the different chronic pretreatment conditions on the steroidogenic phenotype of the cells. Cells were also treated with a range of agonists (VIP, ANP, ATP, 5-HT, VP, ACh, NA; concentrations as stated in chapters 4.0 and 7.0) and the steroidogenic responses of the cell-line characterised.

For these experiments, pretreatment agonists were removed by discarding the medium and washing each well with 2 x 1 ml EBSS. Cells were then incubated in fresh growth medium with agonist or without (basal) for 4 h at 37°C (doses and alternative times as stated in sections 3.2, 4.2 and 7.2). At the end of the treatment period, medium was aspirated from each well and stored at -20°C until assayed for steroid content by RIA as described in section 2.2.4. The remaining monolayer of cells was washed, solubilised and assayed for protein content as described in section 2.2.6. Steroid data was expressed as pmol steroid secreted per mg protein.

2.2.2.3 The effect of pretreatment of H295R cells with forskolin on the subsequent VIP responses.

Cells were treated with growth medium containing forskolin (10 µM) for 72 h (or as stated in time-courses). Medium and agonists were replaced every 24 h. After this time, cells were washed with 2 x 1 ml EBSS and then incubated with growth medium containing VIP for 4 h (concentrations as stated in chapter 4.0). Medium was collected and stored at -20°C until assayed for steroid content by RIAs. Total cellular protein from the remaining cell monolayer was determined (2.2.6).

2.2.2.4 Effect of treatment with VIP, PACAP, RO-25-1553 and the β -adrenoceptor agonists, adrenaline and isoprenaline, on steroid secretion and cyclic nucleotide production by H295R cells

Following pretreatment of cells with forskolin (10 μ M) for 72 h (2.2.2.3), medium was discarded and cells were washed with 2 x 1 ml EBSS and then incubated for 1 h in serum-free growth medium (DMEM / F12 (1:1) containing 1% ITS) prior to the start of the experiment. Cells were then incubated in 1 ml / well fresh serum-free growth medium containing VIP, PACAP, adrenaline or isoprenaline for 4 h at 37°C (concentrations and alternative times as indicated in chapters 4.0 and 5.0). Medium was aspirated from the wells and stored at -20°C until assayed for cortisol or processed as described in 2.2.3.2 and subsequently assayed for cyclic nucleotide content.

2.2.2.5 Effect of β -adrenoceptor antagonists and VPAC receptor antagonists on VIP-stimulated steroid secretion and cyclic nucleotide production by H295R cells

Cells were pretreated with forskolin (10 μ M) for 72 h, washed and incubated in serum-free medium for 1 h prior to treatment with agonists or antagonists (2.2.2.4). Cells were then incubated in serum-free medium alone (basal) or containing antagonist (concentrations as stated in chapter 6.0) for 30 mins prior to the addition of VIP (10^{-9} M or as stated in chapters) for 4 h at 37°C. Medium was aspirated from each well and either stored directly at -20°C until assayed for cortisol or processed as described in 2.2.3.2 and subsequently assayed for cyclic nucleotide content.

2.2.3 Measurement of cyclic nucleotide production

2.2.3.1 In cells

Forskolin-pretreated cells (2.2.2.4) were incubated in serum-free growth medium with or without VIP (3.3×10^{-8} M) for 1, 2.5, 5, 10, 20, 60, 120, 240 min. At each time-point, the medium was rapidly removed (and kept, 2.2.3.2) and 0.5 ml

ice-cold 75% (v/v) ethanol added to each well for a period of 5 mins. Cells were then scraped from the base of the well, using the rubber tip on the plunger from a 1 ml syringe, and removed to an Eppendorf tube. Each well was rinsed with 1 ml ethanol (75% v/v) and this was added to the original 0.5 ml collected. The tubes were centrifuged for 15 mins at 450g in a Heraeus Christ Labofuge B. The cyclic nucleotide containing supernatants were decanted to glass tubes and the ethanol evaporated off under a stream of air at 37°C for 1-2 h on a Techne-Dri block : DB3 fitted with a SC3 Jencons sample concentrator. This extraction method routinely leads to 85-90 % recovery of cyclic nucleotide. Once evaporated to dryness, the residues were reconstituted in 500 µl serum-free growth medium. Samples were immediately acidified, by addition of 5 µl acetic acid (20% v/v) and acetylated by addition of 15 µl of a freshly prepared 2 : 1 mixture of triethylamine / acetic anhydride with immediate vortexing. Samples were stored at -20°C prior to measurement of cAMP or cGMP by RIA (section 2.2.4).

2.2.3.2 *In medium*

Forskolin-pretreated cells (2.2.2.4) were incubated in serum-free growth medium with or without VIP (3.3×10^{-8} M) for 1, 2.5, 5, 10, 20, 60, 120, 240 min (for time-courses, or as detailed in sections 2.2.2.4 and 2.2.2.5). Medium from stimulated cells was removed and divided into 2 aliquots: 350 µl was stored at -20°C for analysis of cortisol content by RIA and the remaining 650 µl was acidified using 6.5 µl acetic acid (20% v/v) for cyclic nucleotide measurement. This acidified aliquot was then subdivided into a 250 µl portion (frozen at -20°C for measurement of cyclic nucleotides using the 'non-acetylated' RIA method) and a 400 µl portion (which was immediately acetylated using 12 µl of a 2 : 1 (v/v) mixture of triethylamine / acetic anhydride and stored at -20°C prior to analysis using the 'acetylated' cyclic nucleotide RIA method).

In some experiments (chapter 5.0), the phosphodiesterase inhibitor IBMX (2-isobutyl-1-methylxanthine, 0.5 mM) was included in the treatment media, to reduce any possible degradation of cyclic nucleotides by phosphodiesterase which could have been present in the cells.

2.2.4 Radioimmunoassays

All radioimmunoassays (RIAs) were performed in polypropylene tubes using a Clinicon Dilutrend automatic diluter. Duplicate tubes were set up for each standard / QC / unknown sample. Tubes containing working tracer solution alone were included in each assay to allow measurement of the 'total counts' added to each tube. Intra-assay drift and inter-assay precision were checked by incorporating quality control (QC) samples in each assay and calculating the coefficients of variation (CVs). The minimum detection limit (MDL) for each assay was calculated by combining and plotting the precision profile data from at least n=10 assays (n=9 assays for cGMP RIA) and reading the concentration of assayed substance at which the CV was $\geq 22\%$ CV (McConway *et al*, 1989).

Following separation of the 'bound' and 'free' tracer fractions, the radioactive pellets in the tubes were analysed in a gamma-counter (LKB 1621 Multigamma) for the times stated and the radioactivity expressed as counts per minute (CPM). Standard curves were fitted using the RIACALC (LKB / Pharmacia) software package.

2.2.4.1 Standard Cortisol RIA

Cortisol secretion into the growth medium by H295R cells was measured using a modification of the cortisol RIA described by Gray *et al* (1983) which employed a double antibody preprecipitate to separate bound and free tracer.

Cortisol standards (1-2000 nM) were prepared by dilution of a stock cortisol solution (10 mM in ethanol) in H295R growth medium and stored at -20°C.

Cortisol tracer (cortisol-3-(O-carboxymethyl) oximino-(2-[¹²⁵I]iodohistamine; obtained in methanol : water (9:1)) was diluted in assay buffer 1 (0.1 M citrate buffer containing 0.2% (w/v) gelatin, 0.013% (w/v) sodium azide, pH 4.0) to obtain a solution with approximately 10⁴ counts / 700 µl (working tracer solution).

The double antibody preprecipitate was prepared from normal sheep serum (530 µl), sheep anti-cortisol (polyclonal primary antibody from SAPU; 650 µl,

reconstituted in assay buffer), 15 ml donkey anti-sheep /goat IgG (secondary antibody) and 10 ml assay buffer. This solution was mixed, left to equilibrate overnight at 4°C, then centrifuged at 230g for 15 mins. The soft pellet was reconstituted in a total volume of 500 ml assay buffer to form the double antibody preprecipitate working suspension.

700 µl working tracer was pipetted into each tube with 100 µl sample / standard / QC. Preprecipitate antibody solution (250 µl) was then added and the tubes vortexed and incubated for 2 h at 37°C. The tubes were centrifuged at 1800g for 30 mins, the supernatants carefully decanted by inversion and the tubes gently blotted on cellulose wadding before reversion. Pelleted antibody-bound counts were measured on the gamma-counter for 120 seconds per tube.

The intra-assay drift was typically <10% and the inter-assay precision was < 10% for the range 50-2000 nM and <15% for the range 10-50 nM. The inter-assay CVs for the QC samples 80 and 800 nM were 7% and 6% respectively. The MDL for the assay was 6 nM. An example of a typical standard curve obtained (fitted to a 4-parameter logistic model) is shown in *Figure 2.1*.

2.2.4.2 More Sensitive Cortisol RIA

When necessary (measurements <50 nM), cortisol secretion from H295R cells was measured using a more sensitive “in-house” modification of the cortisol assay described in section 2.2.4.1. Polyclonal sheep anti-cortisol antibody, obtained from SAPU, was initially used to bind cortisol in the liquid phase. Following an incubation phase, donkey anti-sheep / goat secondary antibody (which bound to the first antibody) was used to separate the bound and free tracer.

Standards were prepared as described in section 2.2.4.1 (either in serum-containing or serum-free growth medium to match the experimental conditions) but in concentrations 0.31-80 nM, with QC samples of 4, 8 and 16 nM. Cortisol tracer (as in 2.2.4.1) was diluted in 100 mM citrate-phosphate buffer, pH 4.0, containing 0.02% (w/v) gelatin and 0.0013% (w/v) sodium azide (assay buffer 2) to give a working tracer solution of approximately 10^4 cpm / 650 µl.



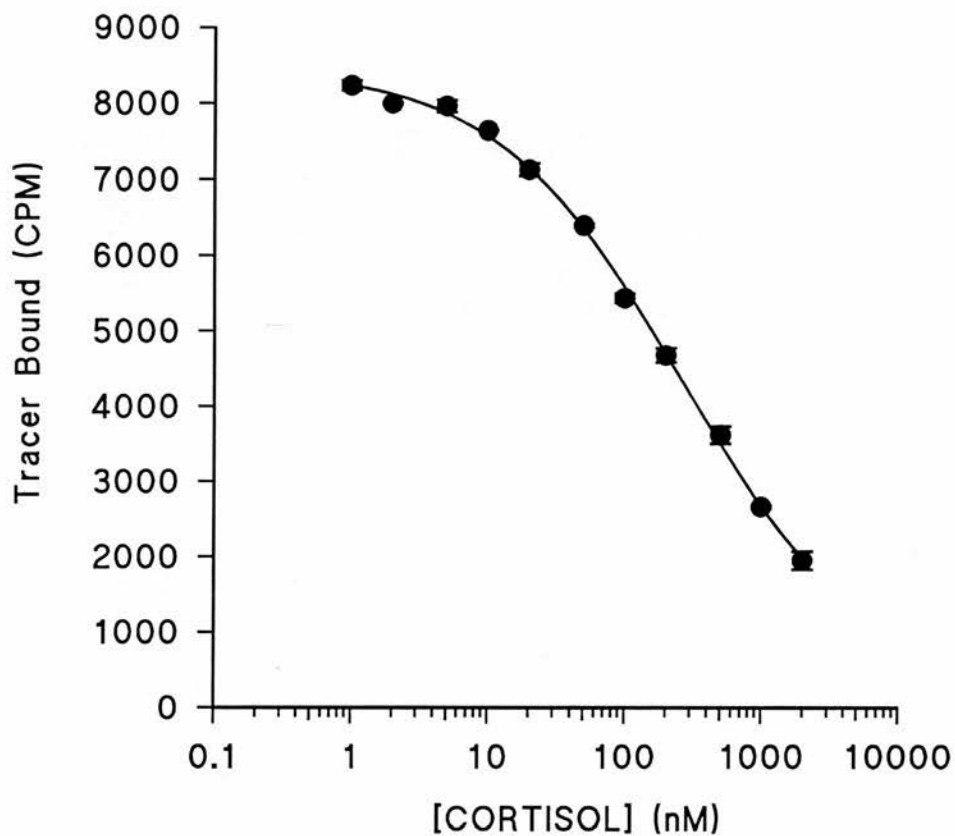


Figure 2.1

Representative standard curve for the 'standard' ^{125}I radioimmunoassay of cortisol. Standard solutions of cortisol (1-2000 nM, in H295R growth medium as used in each experiment) were assayed in duplicate and the standard curve fitted to a four parameter logistic equation. Tracer binding in the absence of cortisol was typically 70-80% of the total tracer added.

Working cortisol tracer (650 μ l) and sample / standard / QC (50 μ l) were pipetted into tubes before addition of the primary antibody (sheep anti-cortisol; initial dilution of 1:5000 in assay buffer 2). The tubes were vortexed and incubated for 4 h at room temperature (20-25°C). 100 μ l each of normal sheep serum (initial dilution of 1:200 in assay buffer 2) and secondary antibody (donkey anti-sheep / goat IgG; initial dilution of 1:15 in assay buffer 2) were then added, the tubes vortexed and incubated overnight at 4°C. Following centrifugation at 1800g, for 30 mins, the supernatant was carefully decanted (as described in section 2.2.4.1) and the tubes counted in the gamma-counter for 120 seconds each.

Intra-assay variation was routinely <10% and inter-assay precision was <10% for the range 10-80 nM. The inter-assay CVs for the QC samples 4, 8 and 16 nM were 15%, 15% and 6% respectively. The MDL was 3 nM. An example of a typical standard curve obtained (fitted to a 4-parameter logistic model) is shown in *Figure 2.2*.

2.2.4.3 Corticosterone RIA

Corticosterone secretion from H295R cells was measured by an “in-house” RIA adapted from a method published by Al-Dujaili *et al* (1981). The method employed the use of a polyclonal antibody to bind corticosterone and a dextran-coated charcoal suspension to separate bound from free tracer.

Standards were prepared in experimental growth medium at concentrations between 0.1-200 nM from a stock solution of 2 mM corticosterone in ethanol. QC samples at concentrations of 1.5, 15 and 150 nM were routinely used in each assay. Aliquots of standards and QCs were stored at -20°C.

A working solution of iodinated corticosterone tracer was prepared by dilution in assay buffer 3 (0.1 M phosphate buffer, pH 7.4, containing 0.1% (w/v) sodium azide and 0.1% (w/v) BSA) to give ~ 4000 cpm / 100 μ l.

50 μ l sample / standard / QC was added to each tube followed by 100 μ l tracer and then 100 μ l of corticosterone antibody (initial dilution 1:8000 in assay buffer 3, as determined by an antibody dilution curve), the tubes vortexed, covered in cling-film and incubated overnight at 4°C. Tubes were placed on ice and 500 μ l ice-

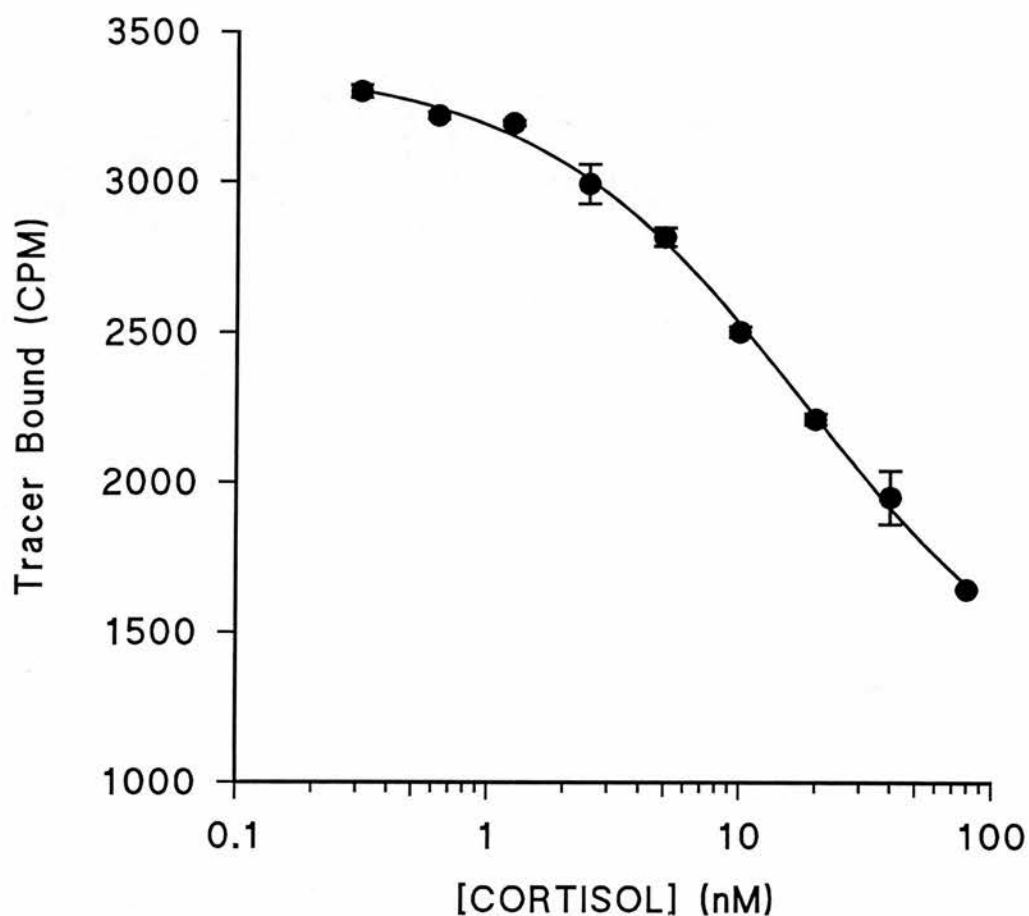


Figure 2.2

Representative standard curve for the more 'sensitive' ^{125}I radioimmunoassay method for measurement of cortisol. Standard solutions of cortisol (1-80 nM, in H295R growth medium as used in each experiment) were assayed in duplicate and the standard curve fitted to a four parameter logistic equation. Tracer binding in the absence of cortisol was typically 40-50% of total tracer added.

cold charcoal suspension (0.6% (w/v) activated charcoal, 0.06% (w/v) dextran T70, 0.04% gelatin in 0.1 M phosphate buffer, pH 7.4) was added to each tube. Following centrifugation at 1800g for 30 mins, 4°C, the supernatant, containing antibody-bound tracer, was carefully decanted and the charcoal pellets, containing the remaining free tracer unbound to antibody, were counted in a gamma-counter for 180 seconds.

Intra-assay drift was routinely found to be <10% and inter-assay CVs for QC samples 1.5, 15 and 150 nM were 22%, 11% and 9% respectively. Overall, the inter-assay precision was <15% for the range 2-50 nM. The MDL for the assay was 1.5 nM. A specimen standard curve (fitted to a 4-parameter logistic model) is illustrated in *Figure 2.3*.

2.2.4.4 Aldosterone RIA

Aldosterone secretion from H295R cells was measured using an “in-house” RIA adapted from Brochu *et al* (1989). The method was essentially similar to that used for the corticosterone RIA, employing a charcoal suspension to separate free from bound tracer.

Standards over the range 0.05-50 nM were prepared in experimental growth medium, from a stock solution of 50 µM aldosterone in ethanol. QC samples were used at 1, 10 and 20 nM. Intra-assay variation was typically <10%. The inter-assay CVs for the QC samples 1, 10 and 20 nM were 23%, 15% and 4% respectively. The MDL could not be calculated as insufficient precision profile data was obtained.

Iodinated aldosterone (aldosterone - 3 (O - carboxymethyl) oximo - (2[¹²⁵I]iodohistamine) was diluted in assay buffer 3 (section 2.2.4(iii)) to give a tracer solution at ~ 4000 cpm / 100 µl.

Tubes were set up as described for the corticosterone assay with 50 µl sample / standard / QC, 100 µl tracer and 100 µl antibody followed by overnight incubation at 4°C. Bound tracer was separated from free tracer by addition of 500 µl charcoal suspension followed by centrifugation (section 2.2.4.3). *Figure 2.4* illustrates a typical standard curve obtained, fitted to a 4-parameter logistic model.

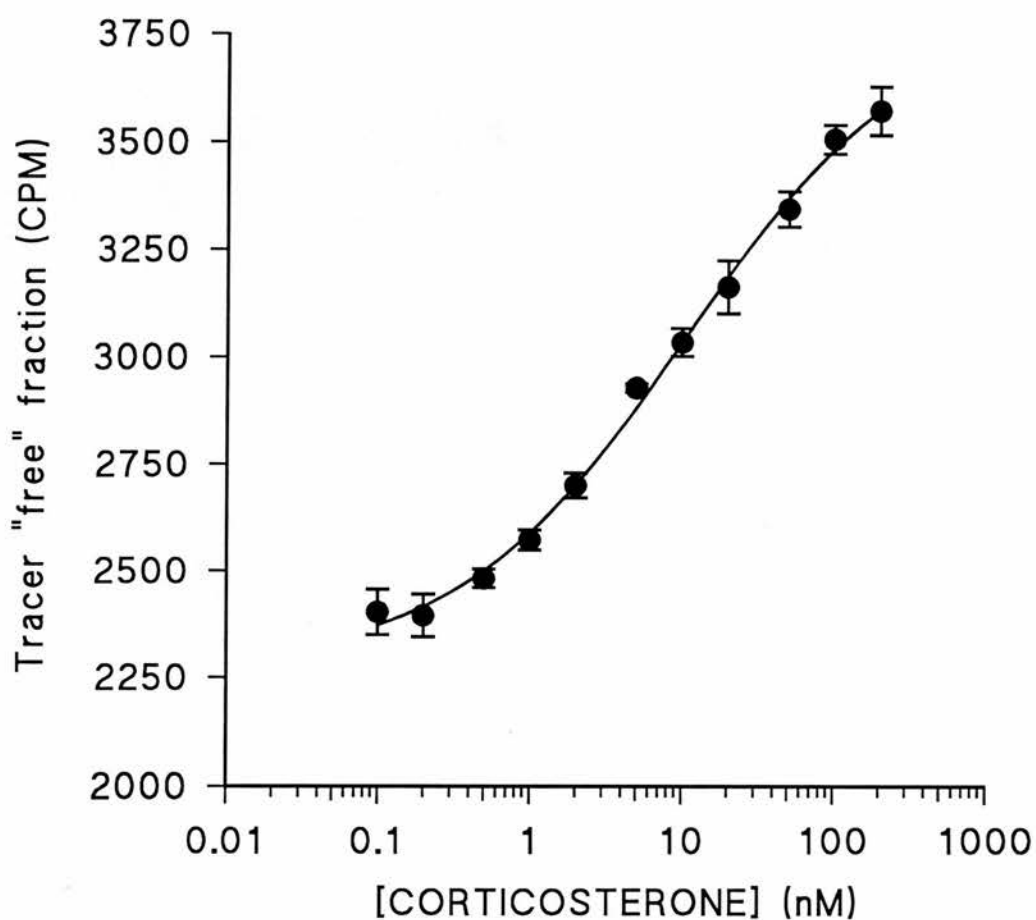


Figure 2.3

Representative standard curve for the ^{125}I radioimmunoassay for corticosterone. Standard solutions of corticosterone (0.1-200 nM, in H295R growth medium as used in each experiment) were assayed in duplicate and the standard curve fitted to a four parameter logistic equation. Tracer binding in the absence of corticosterone was typically 50-60% of total tracer added.

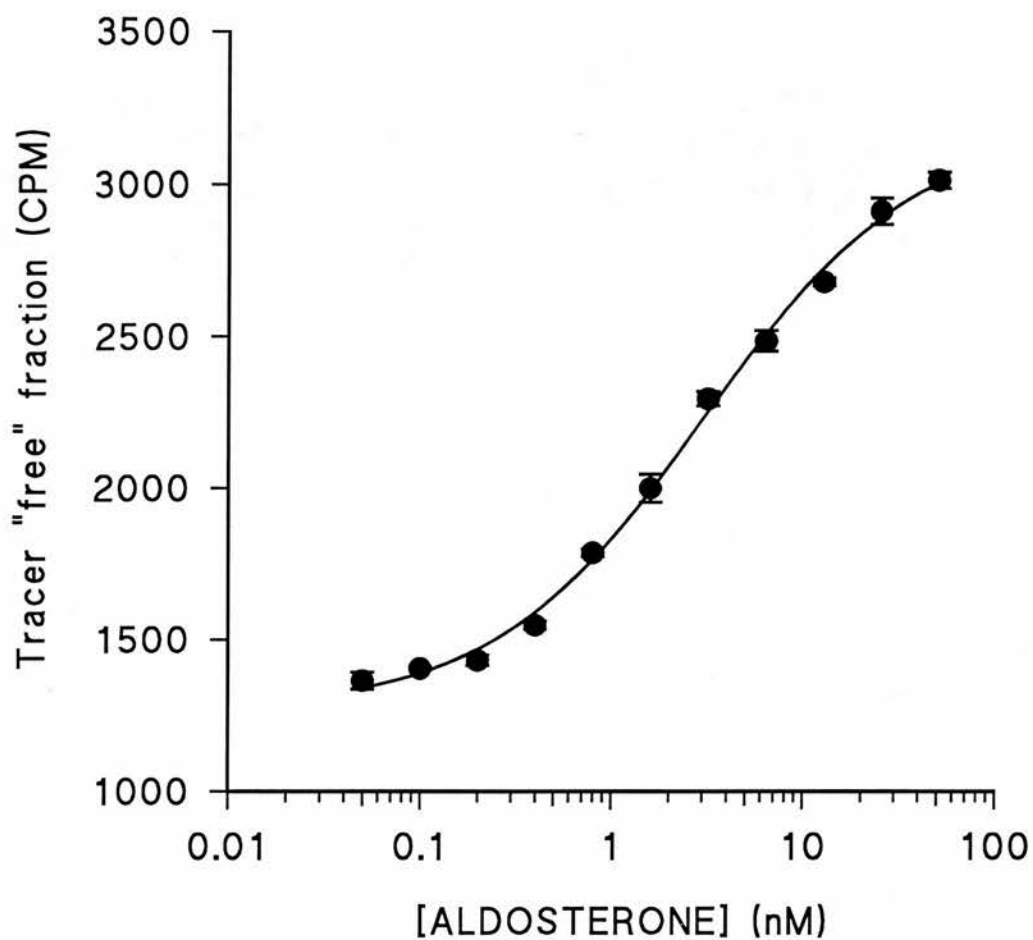


Figure 2.4

Representative standard curve for the ^{125}I radioimmunoassay of aldosterone. Standard solutions of aldosterone (0.05-52 nM, in H295R growth medium as used in each experiment) were assayed in duplicate and the standard curve fitted to a four parameter logistic equation. Tracer binding in the absence of aldosterone was typically 65-75% of total tracer added.

2.2.4.5 Androstenedione RIA

The RIA used to measure androstenedione secretion from H295R cells was an adaptation of the method by Thomson *et al* (1989) and employed the use of a second antibody precipitation to separate bound from free tracer.

Standards were prepared in experimental growth medium representing 0.5-35 nM. QC samples were used at 1.1 and 3.5 nM. Intra-assay drift was routinely found to be <10% and inter-assay variation was <10% for the range 0.8-3.5 nM and <15% for the range 0.5-6 nM. Inter-assay CVs for the QC samples 1.1 and 3.5 nM were 8% and 13% respectively. The MDL was 0.5 nM.

Radiolabelled androstenedione (androstenedione-3-carboxymethyloxime [¹²⁵I]iodohistamine) was diluted in assay buffer 4 (0.1 M phosphate buffer, pH 7.4, containing 0.25% (w/v) BSA) to give a tracer solution at 10⁴ cpm / 150 µl.

Into duplicate tubes was pipetted 50 µl sample / standard / QC, 150 µl tracer solution and 200 µl androstenedione antibody (initial working dilution ~1:20,000, determined by an antibody dilution curve, prepared in assay buffer 4). Tubes were vortexed, covered with cling-film and incubated at room temperature (20-25°C) for 2 h.

Following the addition of 400 µl second antibody preprecipitate solution (donkey anti-rabbit (1:40 dilution) and normal rabbit serum (1:250 dilution), prepared in assay buffer 4), tubes were vortexed and incubated overnight at 4°C. Tubes were centrifuged at 1800g for 45 mins, 4°C, and the supernatant decanted as described in section 2.2.4.1. Pellets, containing antibody-bound tracer, were counted on a gamma-counter for 120 seconds. *Figure 2.5* shows a typical standard curve obtained (fitted to a 4-parameter logistic model).

2.2.4.6 Cyclic nucleotide RIAs

The RIA methods used to measure cAMP and cGMP production by H295R cells were based on the method of Brooker *et al* (1979). The antibodies used were raised in rabbits against the succinylated form of the cyclic nucleotide and the radiolabel was the succinylated tyrosine methyl ester of the cyclic nucleotide. Thus,

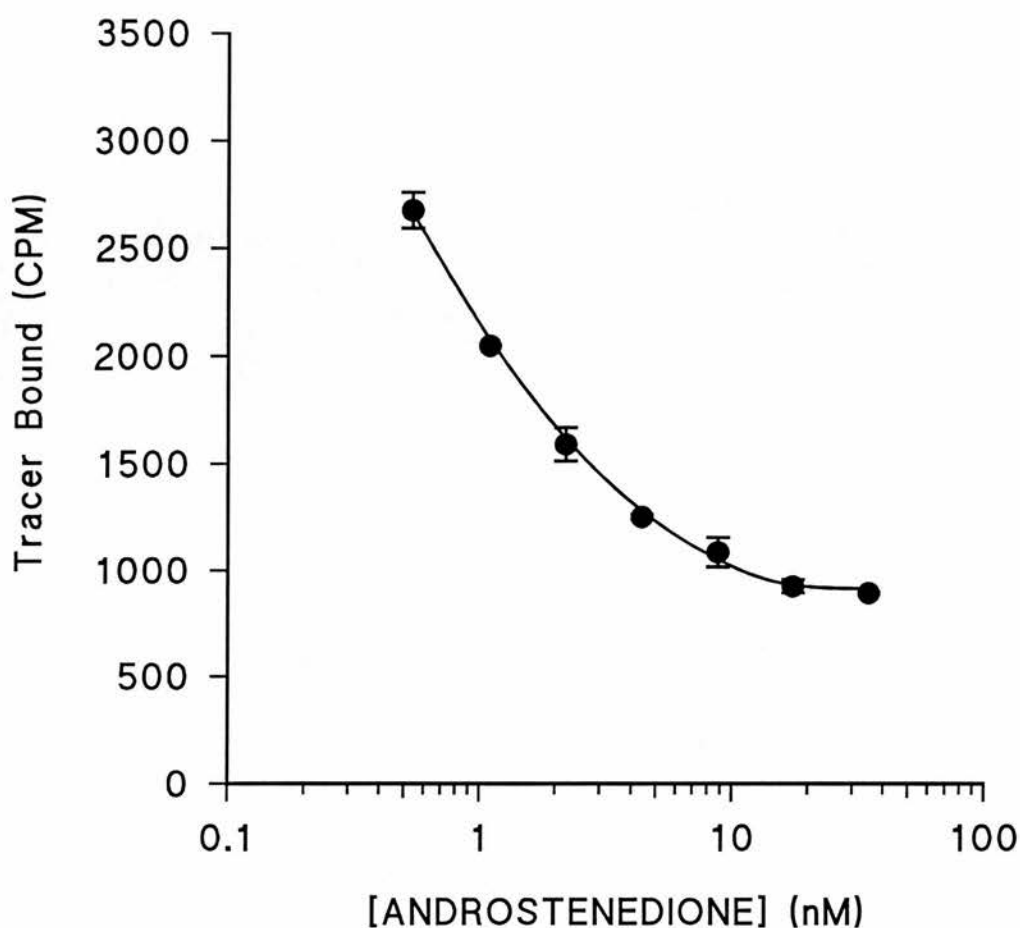


Figure 2.5

Representative standard curve for the ^{125}I radioimmunoassay for androstenedione. Standard solutions of androstenedione (0.5-35 nM, in H295R growth medium as used in each experiment) were assayed in duplicate and the standard curve fitted to a four parameter logistic equation. Tracer binding in the absence of androstenedione was typically 55-65% of total tracer added.

pre-acetylation of standards and samples gave an increased assay sensitivity. Depending on the sensitivity required in the RIA, cyclic nucleotides were measured by either the 'non-acetylated cyclic nucleotide RIA' (the case for cAMP analysis) or the 'acetylated cyclic nucleotide RIA' (the case for cGMP analysis).

(a) *Non-acetylated cyclic AMP RIA*

Standards were prepared from a stock solution of 32 μM of the sodium salt of cAMP in acidified serum-free growth medium (20% (v/v) acetic acid 1: 99 serum-free growth medium) over the range 1.25-320 nM. QC samples were used at 5, 20 and 80 nM. Intra-assay variation was <12% and inter-assay drift was <10% for the range 15-200 nM and <15% for the range 5-320 nM. The MDL was 2 nM.

100 μl working tracer solution (containing ~ 6000 cpm/100 μl , diluted in 0.05 M acetate buffer, pH 5.0, 0.1% (w/v) gelatin) was combined with 50 μl sample / standard / QC and 100 μl antibody working solution (initial titre determined by performing an antibody dilution curve with each new batch of tracer, usually $\sim 1:20,000$) and incubated overnight at 4°C.

The separation of antibody-bound and -free tracer fractions (using a dextran-coated charcoal suspension) and subsequent analysis of radioactive content of the tubes was performed as described in section 2.2.4.3. An example of a typical standard curve obtained (fitted to a 4-parameter logistic equation) is shown in *Figure 2.6*.

(b) *Acetylated cyclic GMP RIA*

Standards (over the range 0.125-32 nM), QCs (0.5, 5 and 10 nM) and samples were pre-acetylated in glass tubes (as described in 2.2.3.2). Intra-assay drift was routinely found to be <10% and inter-assay variation was <10% for the range 0.7-9 nM and <15% for the range 0.4-20 nM. Inter-assay CVs for the QC samples 0.5, 5 and 10 nM were 14 %, 8 % and 10 % respectively. The MDL was 0.25 nM (see chapter 5.0).

100 μl sample / standard / QC was combined with 150 μl iodinated cGMP tracer (working solution ~ 6000 cpm/150 μl , diluted in 0.05 M acetate buffer, pH 5.0,

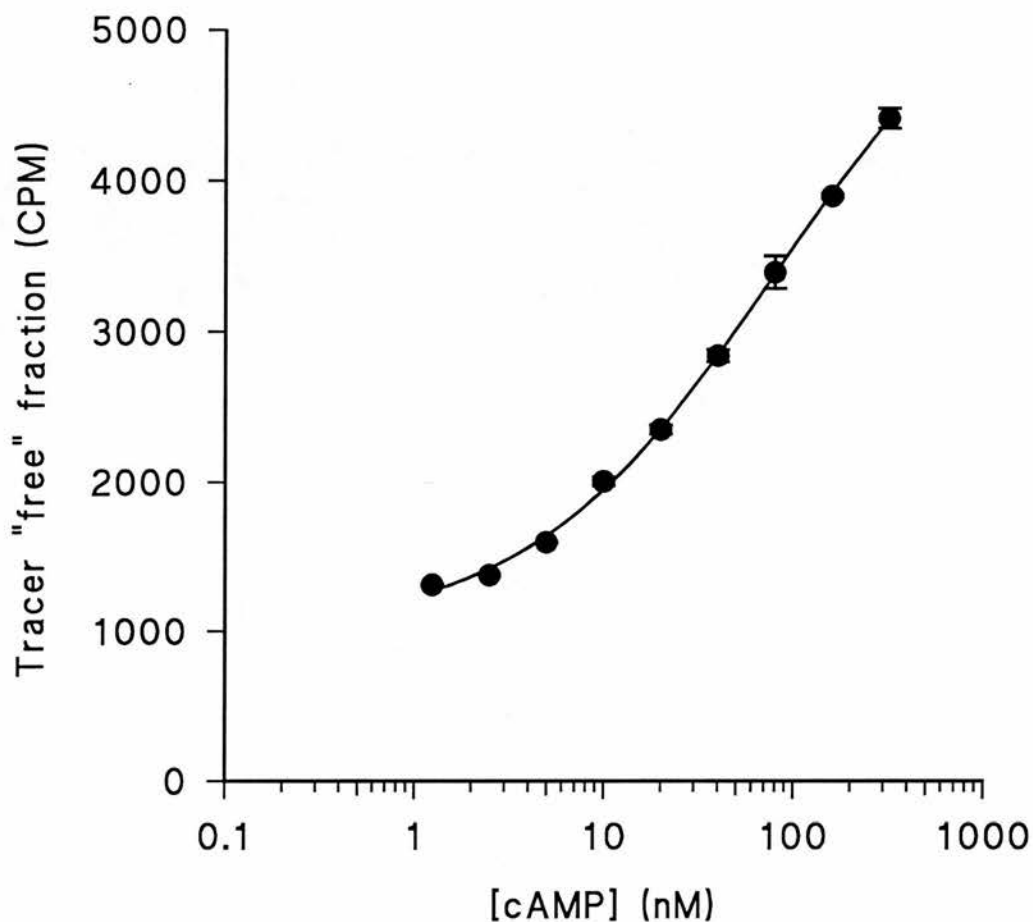


Figure 2.6

Representative standard curve for the ^{125}I radioimmunoassay for cAMP. Standard solutions of the sodium salt of cAMP (1-320 nM, in acidified H295R growth medium, pH 5.0) were assayed in duplicate and the standard curve fitted to a four parameter logistic equation. Tracer binding in the absence of cAMP was typically 70-80% of total tracer added.

0.1% (w/v) gelatin) and 100 μ l antibody (initial titre determined from an antibody dilution curve) and incubated overnight at 4°C.

A dextran-coated charcoal suspension was used to separate bound and free tracer fractions (as described in 2.2.4.3). *Figure 2.7* shows a typical standard curve obtained (fitted to a 4-parameter logistic model).

2.2.5 Measurement of total [3 H] phosphoinositol

Stimulation of phosphatidylinositol turnover was measured in H295R cells. The method used is described by Bird *et al* (1992).

H295R cells were seeded into 12-well plates and grown to near-confluence. Cells were then pretreated with growth medium with or without forskolin (10 μ M) for 24 h (1 ml / well), then in serum-free growth medium with the addition of [3 H]inositol (10 μ Ci / ml) and forskolin (10 μ M) for a further 48 h (0.5 ml / well). Before subsequent acute treatment with agonists, labelled medium was removed and the cells incubated at 37°C, for 15 mins, with 0.5 ml DMEM / F12 supplemented with 0.2 % (w/v) BSA to wash away extracellular inositol. This medium was then removed and replaced with 0.45 ml DMEM / F12 containing 0.2% BSA, 10 mM unlabelled inositol (to chase unincorporated labelled inositol out of the cells) and 10 mM LiCl (to inhibit phosphoinositol phosphatases). After 15 mins, additions of 50 μ l DMEM / F12, 0.2% BSA were added with or without agonists and the cells incubated at 37°C for 30 mins. At the end of this time, 250 μ l ice-cold perchloric acid (15% v/v) was added to each well to denature proteins and lyse the cells.

The cells were then scraped from the base of the wells, using the rubber tip from the plunger of a 1 ml syringe, and the contents of the well removed, with a 0.5 ml water wash, to a 1.5 ml Eppendorf tube. The samples were centrifuged at 3300g in a microfuge (Scotlab microcentaur) for 3 mins and the supernatants (containing free inositol and phosphoinositols) transferred to glass tubes. A mixture of 1:1 (v/v) trichlorotrifluoroethane / tri-n-octylamine was freshly prepared and 1.5 ml added to each tube, and the samples vortexed until the contents appeared milky. Following centrifugation (Beckman GS-6R benchtop centrifuge) at 3000 rpm, for 3 mins, the

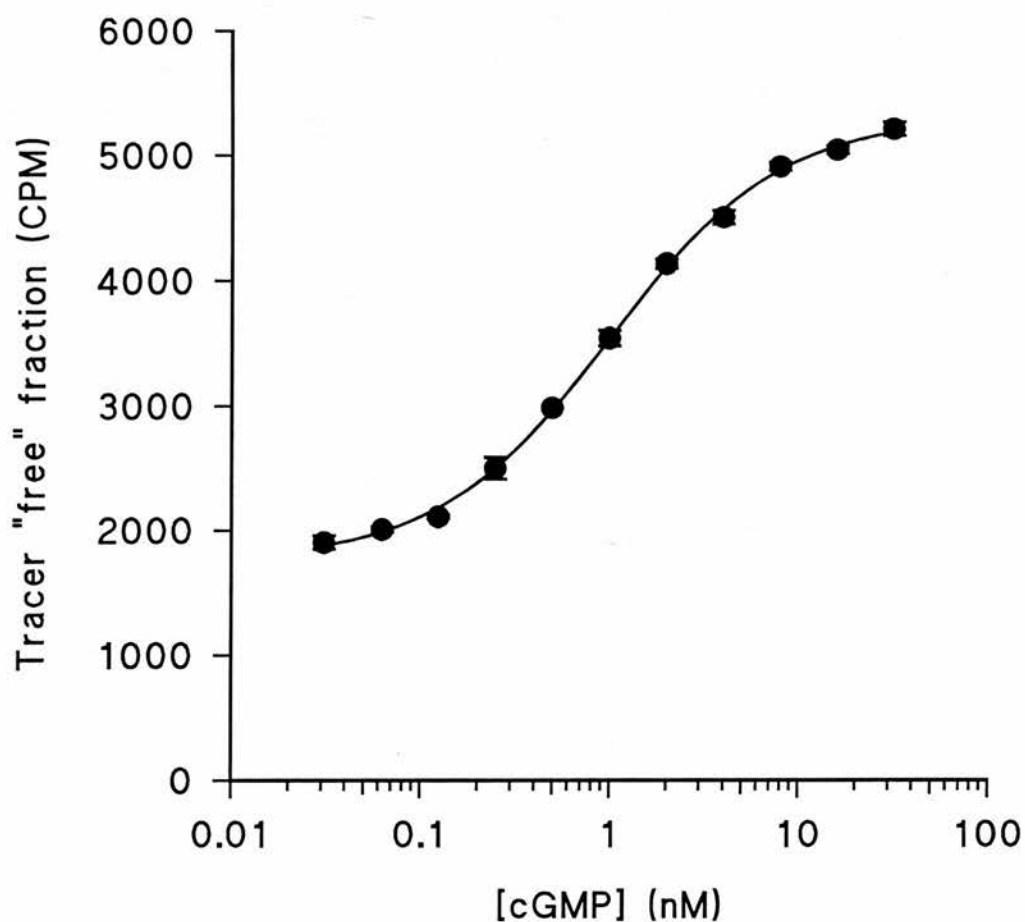


Figure 2.7

Representative standard curve for the ^{125}I radioimmunoassay for cGMP. Standard solutions of the sodium salt of cGMP (0.1-32 nM, in acidified H295R growth medium, pH 5.0) were acetylated (as described in section 2.2.3.2), assayed in duplicate and the standard curve fitted to a four parameter logistic equation. Tracer binding in the absence of acetylated-cGMP was typically 60-70% of total tracer added.

upper aqueous phase (0.9 ml) was removed to a fresh Eppendorf tube and stored at -20°C prior to assay.

Columns for phosphoinositols extraction were prepared by loading 0.5 ml of a 1:1 AGI-X8 anion exchange resin / distilled water suspension into polypropylene columns with a 1 cm diameter scintered glass base. The gel bed was washed through with 4 ml distilled water prior to sample loading.

Samples (0.9 ml) were thawed, 100 µl EDTA (10 mM) added to each and then loaded onto the columns. The columns were washed with 2 x 4 ml distilled water to remove unbound inositol. The total phosphoinositols were then eluted with 2 x 2 ml ammonium formate (1 M) / formic acid (0.1 M) buffer into scintillation vials (2 vials per sample). 3 ml scintillant (HydroLuma) was added to each vial and shaken well to mix the phases. The radioactivity of the samples (in disintegrations per minute, DPM) was determined by counting for 10 minutes in a Canberra-Packard CA1900 liquid scintillation counter.

2.2.6 Protein assay

The cellular protein content of each well was determined by the dye-binding technique of Bradford (1976) adapted for use on a Cobas Fara (Roche Diagnostics, Welwyn Garden City, UK) centrifugal analyser.

Working Coomassie Brilliant Blue G-250 was prepared as described by Bradford (1976) although it was filtered, before use, through Whatman grade 1 filter paper.

Wells were washed with 2 x 1 ml EBSS and the cells solubilised in 0.5 ml NaOH (0.3 M) / SDS (0.1% w/v) and removed to a polypropylene tube. Wells were rinsed with 0.5 ml distilled water and this wash added to the tube. Samples were diluted 1:10 with distilled water prior to analysis. Assay standards covering a range of concentrations of BSA (0-100 µg/ml) were used to construct a standard curve.

2.2.7 Molecular Biology methods

In order to demonstrate the presence of VIP receptor mRNA, H295R cells were grown to near-confluence in 75 cm² flasks. Cells were treated for 72 h in growth medium with or without forskolin (10 µM) (medium and agonists replaced every 24 h) prior to extraction of total RNA.

2.2.7.1 Isolation of total RNA from H295R cells

In order to ensure all items used in the extraction of RNA from cells were RNase free, sterile, disposable plasticware was used wherever possible. Glassware was washed and baked overnight at 160°C and non-sterile plasticware washed in DEPC-treated water for 2 h at 37°C, rinsed and autoclaved. Wherever possible, solutions (prepared in RNase-free glassware with autoclaved water) were treated with 0.1% DEPC for at least 12 h and then autoclaved. Gloves were worn throughout the procedure.

The method used was an adaptation of the RNeasy method described by Chomczynski & Sacchi (1987). Briefly, cells were rinsed with EBSS (warmed to 37°C) to remove traces of growth medium, then lysed with 2 ml RNeasy per 75 cm² flask. Using a cell-scraper, cells were detached from the bottom of the flask and the viscous mixture pipetted up and down several times to ensure solubilisation of the RNA, then transferred to 2 x 1.5 ml Eppendorf tubes (1 ml per tube) and placed on ice. Following the addition of 100 µl of chloroform, tubes were vortexed for 15 seconds, left on ice for 5 mins, then centrifuged in a microfuge at 13000 rpm, 4°C, for 15 mins. The upper aqueous phase was carefully removed to a fresh Eppendorf tube, avoiding the protein interphase layer. The RNA was precipitated by the addition of 1 volume of isopropanol, mixed by inversion and incubated at 4°C for 30 mins. RNA was then pelleted by centrifugation (13000 rpm, 4°C, 15 mins) and the supernatant discarded. The pellet was washed with 180 µl 70% (v/v) ethanol, vortexed briefly and re-centrifuged. After removal of supernatant, the pellet was air-dried for 10-15 mins prior to resuspension in 50 µl DEPC-treated water. Samples were stored at -70°C.

The concentration and purity of RNA in each sample was determined by measuring the A_{260} ($1 \times A_{260}$ unit = 40 μg RNA) and the A_{260} / A_{280} ratio, respectively, in a UV spectrophotometer.

2.2.7.2 Detection of VIP and PACAP receptor mRNAs in H295R cells by reverse transcription-polymerase chain reaction (RT-PCR)

These methods were initially performed with the assistance of Dr. Eve Lutz, MRC Brain Metabolism Unit, Royal Edinburgh Hospital, who also kindly provided the VPAC1 receptor, VPAC2 receptor, PAC1 receptor and HPRT primers and positive control material.

(a) Reverse transcription (RT) of total RNA to obtain cDNA

The method used was that described in the Stratagene RT-PCR kit manual. 5-10 μg total RNA (diluted in DEPC-treated water to a volume of 38 μl) was reverse transcribed in a 50 μl reaction volume. Following the initial addition of 3 μl random primers (100 ng/ μl) to the RNA solution, the mixture was heated to 65°C for 5 mins, then cooled slowly to room temperature, to denature the secondary structure of the RNA and to allow the primers to anneal. The following reagents were then added:

- 5 μl of 10x first strand buffer
- 1 μl of RNase Block Ribonuclease Inhibitor (40 U/ μl)
- 2 μl of 100 mM dNTPs
- 1 μl of MMLV-RT (reverse transcriptase; 50 U/ μl).

The tubes were vortexed and incubated at 37°C for 1 h followed by 5 mins at 90°C to denature the MMLV-RT.

(b) Polymerase Chain Reaction (PCR)

The polymerase chain reaction was used to amplify specific sequences for the VPAC1 receptor, VPAC2 receptor and PAC1 receptor. Primer sets used for each receptor were as follows:

VPAC1 receptor:

5'-(CTGCTGATCCCCCTGTTTGGAGTA)-3' (forward)

5'-(CTACCGTGGTGTGGTGCTGGTG)-3' (reverse)

VPAC2 receptor:

5'-(TGCCCTGGTGGGTCATACGAA)-3' (forward)

5'-(AATAGGTTTCCGGTCCCATTTCAG)-3' (reverse)

PAC1 receptor:

5'-(ATTGGCATTATCGTCATCCTTGTG)-3' (forward)

5'-(AGAGACGGGTGTCGGTGCTTG)-3' (reverse)

HPRT primers were included as a control:

5'-(GCTSCACTGCMCIAGIAACTWCATCCAC)-3'(forward)

5'-AARCAGTAIAGIAYIGCIACMAYIARCCCTGGAA)3'(reverse)

(where S=G or C, M=A or C, W=A or T, R=A or G, Y=C or T and I=inosine)

Primers were designed using the primer design program available from the UK Human Genome Mapping Project Resource Centre Computing Facilities (web page: <http://www.hgmp.mrc.ac.uk/>). All the primers used were intron-spanning.

Positive control material used for each receptor was:

VPAC1 receptor: λ gt 10 lung cDNA (human);

VPAC2 receptor: human placental cDNA;

PAC1 receptor: λ gt 10 brain cDNA (human).

Negative control tubes containing each set of primers but no DNA (5 μ l DEPC-treated water instead) were included.

cDNA (5 μ l) from the reverse transcription reaction was amplified in a 100 μ l reaction volume containing:

10 μ l of 10 X DNA polymerase buffer

0.8 μ l of 100 mM dNTPs

1 μ l of 15 μ M primer set

82.7 μ l DEPC-treated water

0.5 μ l *Taq* DNA polymerase (5 U/ μ l) (added after initial heat denaturation : simplified 'hot start' conditions)

The 'standard' reaction buffer used was 10 mM Tris HCl, 50 mM KCl, 1.5 mM MgCl₂, pH 8.8, though a buffer containing 10 mM Tris HCl, 25 mM KCl, 3.5 mM MgCl₂, pH 8.3, was used once during optimisation of the VPAC1 receptor PCR procedure.

25 μ l light mineral oil was layered on top of each reaction tube and reaction carried out in a Perkin Elmer thermal cycler. The reaction conditions used were: 91°C for 5 mins (initial heat denaturation step), then a run of 35 cycles of:

91°C for 1 min (denaturation)

65°C for 1min (annealing)

72°C for 3 mins (extension)

followed by a final elongation step at 72°C for 10 mins. For optimisation of the procedure the following were varied: cycle number (30, 35 and 40), annealing temperature (65°C and 72°C).

(c) Analysis of PCR products by agarose gel electrophoresis

10 μ l of PCR product (from below the oil layer) was combined with 2 μ l gel loading buffer and loaded onto an ethidium bromide-containing 0.8% (w/v) agarose gel and electrophoresed alongside 123 bp DNA molecular weight markers. The PCR product was visualised under ultraviolet trans-illumination.

2.2.8 Statistical analyses of data

Statistical analyses of data were performed using the Student's t-test with Bonferroni correction and data were considered significantly different from basal data (or as stated) at a P-value of less than 0.05. P-values are quoted as * $P < 0.05$, ** $P < 0.02$, *** $P < 0.01$ and $P \geq 0.05$ (non-significant).

3.0 Steroid responses of H295R cells to All and forskolin and alteration of the steroid phenotype of the cell-line

3.1 Introduction

3.1.1 Mechanism of steroidogenic action of ACTH in the adrenal

ACTH can regulate steroid production indirectly, for example, by increasing blood flow rate through the adrenal (section 1.6.3) and directly, by stimulating ACTH receptors on adrenocortical cells (Schimmer *et al*, 1968). ACTH stimulation of adrenocortical steroidogenesis is considered to be mainly mediated via the second messenger adenosine 3' 5' cyclic monophosphate (cAMP) (Schimmer *et al*, 1995, for review). An increase in intracellular cAMP production can be artificially created using the adenylate cyclase activator, forskolin (section 5.1.1).

Extracellular calcium is also important in transducing the ACTH signal since ACTH-induced aldosterone secretion is inhibited in human adrenal glomerulosa cells in calcium-free conditions (Gallo-Payet *et al*, 1996) and the calcium chelator, EGTA, inhibited ACTH-induced steroid synthesis in bovine zfr cells (Kimoto *et al*, 1996) and ACTH-induced protein kinase activity in adrenal cells (Watanabe *et al*, 1997).

The actions of ACTH on steroid production can be described in terms of an 'acute' effect (taking place within a few minutes to hours) and a 'chronic' effect (taking place over hours-days).

3.1.1.1 Acute action of ACTH

This effect generally involves an increase in cholesterol supply to the inner mitochondrial enzyme P450_{scc}, which catalyses the first step in all adrenal steroid biosynthesis. The increase in substrate availability results in increased enzyme activity and increased steroidogenesis.

Following receptor activation, ACTH causes an increase in cholesteryl ester conversion to free cholesterol, by increasing the activity of the enzyme cholesteryl ester hydrolase via cAMP-dependent protein kinase phosphorylation (Beckett &

Boyd, 1977). In addition, ACTH increases the rate of transport of cholesterol across the inner mitochondrial membrane and binding to P450_{scc}. This process involves two steps: transport of cholesterol to the outer mitochondrial membrane and translocation from the outer membrane to the inner mitochondrial membrane. The first step is thought to involve components of the cytoskeleton. ACTH may facilitate changes in the cytoskeletal structure that result in lipid droplets and mitochondria achieving contact by activating protein kinases, including cAMP-dependent-protein kinase A (PKA) and protein kinase C (PKC) (reviewed by Hall & Almahbobi, 1997). The second, cycloheximide-sensitive, step involves a labile protein and results in transfer of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane. The identity of this protein is still debated but candidates include StAR (Steroidogenic Acute Regulatory) protein, SCP2 (Sterol Carrier Protein 2), SAP (Steroidogenesis Activator Polypeptide) or PBR / DBI (Peripheral Benzodiazepine Receptor and its intracellular ligand, Diazepam Binding Inhibitor) (Stocco & Clark, 1996 for review).

3.1.1.2 Chronic action of ACTH

The chronic phase is characterised by effects of ACTH on steroidogenic enzyme expression in the adrenal cortex. The importance of ACTH in maintaining steroid enzyme levels in adrenocortical cells was first established from studies with hypophysectomised animals. Hypophysectomy resulted in a dramatic decrease in P450 enzyme levels in the rat adrenal cortex which could be restored upon administration of ACTH (Purvis *et al*, 1973). It has subsequently been shown that ACTH increases steroidogenic enzyme mRNA synthesis and translation via a cAMP-dependent mechanism (Simpson & Waterman, 1988). The gene for each steroidogenic enzyme appears to have its own regulatory system for cAMP-responsive transcription (Waterman & Bischoff, 1997). ACTH and cAMP have been shown to increase the expression of several adrenal steroidogenic enzymes including P450_{scc}, P450_{c17}, P450_{11 β} and P450_{c21} (Simpson & Waterman, 1988).

Typically, an increase in enzyme transcripts is not seen until approximately 6 h after ACTH treatment of the cells, with increase in enzyme activities a further 6-12 h later (Hanukoglu *et al*, 1990). The circadian nature of ACTH stimulation of the adrenal cortex in non-stress conditions probably results in a maintenance of levels of steroidogenic enzymes rather than causing fluctuations in enzyme levels.

3.1.2 Mechanism of steroidogenic action of AII in the adrenal

AII is the major stimulus to aldosterone secretion from the adrenal cortex. It binds and activates specific AII receptors on zg cells, leading to activation of phospholipase C (PLC) and subsequent formation of inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG) (section 5.1.3) and an increase in intracellular calcium concentration. A rapid rise in intracellular calcium has been correlated with an increased production of aldosterone (reviewed by Ganguly & Davis, 1994). The increase in intracellular calcium concentration is biphasic; a rapid transient rise is followed by a sustained increase. The initial rise in cytosolic calcium has been attributed primarily to IP₃-mediated release from intracellular calcium with the secondary, lower but sustained, increase dependent upon influx across the plasma membrane, probably through voltage-gated calcium channels (see Ganguly & Davis, 1994). DAG is required for the activation of protein kinase C (PKC), by increasing the affinity of the enzyme for phospholipids at physiological calcium concentrations (Nishizuka *et al*, 1984). Activation of PKC in the steroidogenic action of AII is important, as demonstrated by the use of phorbol esters, for example, phorbol myristate acetate (PMA), which activate PKC. However, it has been suggested that calcium-calmodulin may actually be more important than PKC in the stimulation of steroid secretion (Ganguly & Davis, 1994).

AII also stimulates formation of 12-HETE (12-hydroxyeicosatetraenoic acid), a product of the lipoxygenase pathway of arachidonic acid metabolism, in human zg cells and this has been linked to aldosterone synthesis (Natarajan *et al*, 1988a). It has

been suggested that the arachidonic acid required for this pathway is derived from DAG (Natarajan *et al*, 1988b).

AII stimulates PLC activity and concomittant steroid production from bovine and human zfr cells but not from rat zfr cells. This is in keeping with the lack of detectable AII receptors on rat zfr cells. AII can also alter expression of certain steroidogenic enzymes in cultured human adrenal cells, in particular, it increases expression of P450aldo (Aguilera *et al*, 1980; Kramer *et al*, 1980).

The aims of this chapter were to examine the steroid responses of H295R cells to chronic (up to 96 h) and short-term (4 h) treatment with AII, the principal physiological mineralocorticoid agonist, and forskolin, a direct activator of adenylate cyclase. Forskolin was used instead of ACTH, the principal regulator of glucocorticoid secretion *in vivo*, because of reported relatively poor responses of these cells to ACTH (perhaps due to low ACTH receptor density) compared with activators of protein kinase A (Rainey *et al*, 1993).

Of particular interest in these studies was the effect of chronic pretreatment with either AII or forskolin on 1) the overall steroidogenic responsiveness of the cells to subsequent (4 h) agonist stimulation and 2) the alteration of steroidogenic phenotype. The ability to alter the steroidogenic phenotype of the cell-line would allow the effects of VIP (and other agonists) on zona glomerulosa-like and zona fasciculata/reticularis-like cells to be determined.

3.2 Results

3.2.1 Steroid responses of cells to chronic treatment with AII or forskolin

The time-dependent chronic actions of AII and forskolin on cortisol, corticosterone and androstenedione secretions were studied.

Both AII and forskolin were found to cause concentration-dependent increases in cortisol secretion (see *Figure 3.1*). A concentration of 1 nM AII caused a statistically significant increase ($P < 0.01$) in 2 out of 3 experiments and a concentration of 10 nM AII caused a statistically significant ($P < 0.01$) increase in 3 out of 3 experiments, triplicate incubations performed in each experiment. Forskolin caused significant stimulation of cortisol secretion at all three concentrations ($P < 0.01$) (triplicate incubations performed in a single experiment). Concentrations of 10 nM AII and 10 μ M forskolin were used in later experiments.

The chronic effects of AII or forskolin treatment were investigated by stimulating cells with these agonists for 24, 48, 72 or 96 h. Media and agonists were replaced every 24 h so that only the medium from the last 24 h of each treatment period was collected for steroid assays. *Figure 3.2* shows the different cortisol, corticosterone and androstenedione secretion profiles obtained. Response Ratios (RR, response to stimulus / basal secretion) for individual experiments are presented in *Table 3.1*. Chronic treatment of the cells with AII resulted in a time-dependent increase in cortisol and corticosterone secretion. Maximum cortisol output and RR were observed after 72 h AII treatment (mean RR after 72 h = 5.9 (± 1.7)-fold, $n=5$ individual experiments). Maximum corticosterone output and RR were observed after 48-72 h AII treatment (RRs = 6.8-fold and 11-fold in $n=2$ individual experiments, triplicate incubations performed in each), thereafter declining. Androstenedione secretion did not significantly alter over the course of the chronic incubation ($P \geq 0.05$, calculated from triplicate incubations in each of 2 separate experiments) (*Figure 3.2* and *Table 3.1*).

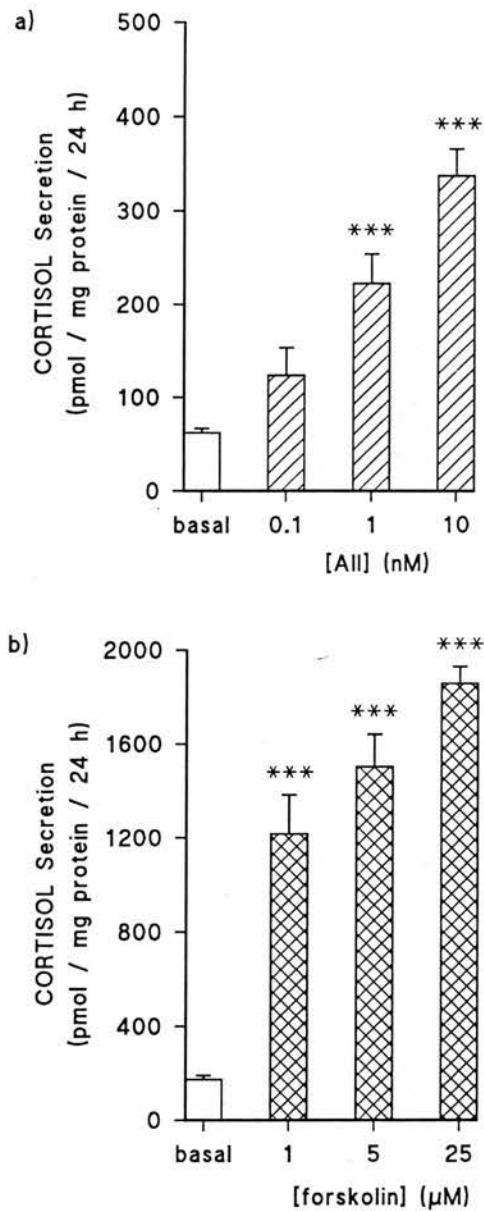


Figure 3.1

Dose-dependent cortisol secretion from H295R cells in response to a) AII or b) forskolin. Cells were treated for 24 h with fresh growth medium alone (basal, open bar) or medium containing AII (0.1, 1 or 10 nM, hatched bars) or forskolin (1, 5 or 10 μM, cross-hatched bars). At the end of this time, medium was removed and assayed for cortisol content by RIA. Cells were solubilised and assayed for total protein content. Results are expressed as mean (\pm s.d.) of data from triplicate incubations and are representative of those obtained in n=3 experiments for AII, n=1 experiment for forskolin. Significant stimulation of cortisol secretion, compared with basal cortisol secretion, is indicated at ***P<0.01. Doses of 10 nM AII and 10 μM forskolin were chosen as treatment doses for subsequent experiments.

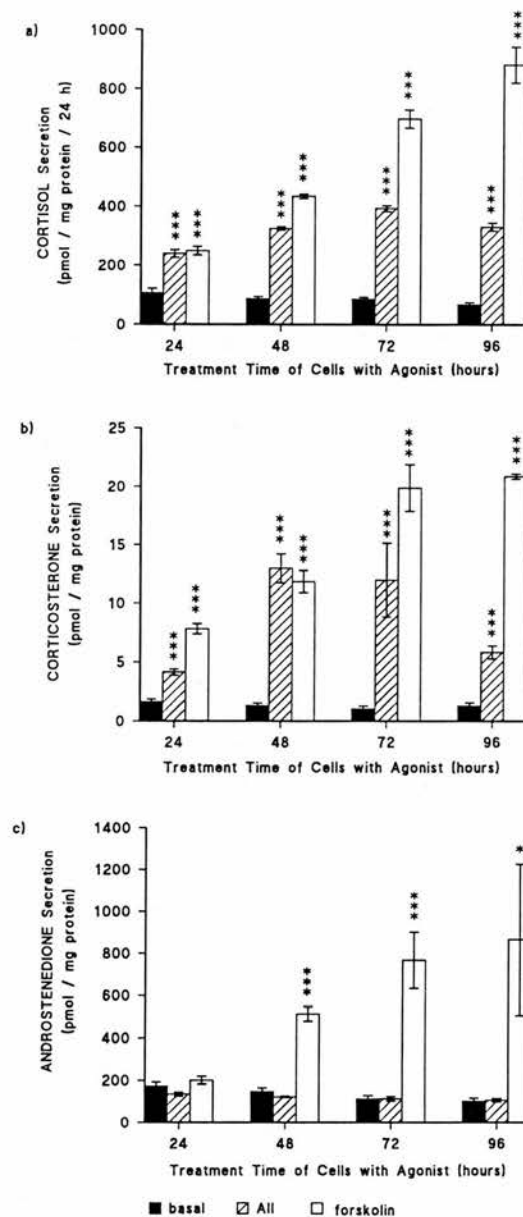


Figure 3.2

Secretion of a) cortisol, b) corticosterone and c) androstenedione from H295R cells in response to chronic treatment with AII or forskolin. Cells were treated with fresh growth medium alone (basal, solid bars) or containing AII (10 nM, hatched bars) or forskolin (10 μ M, open bars) for 24, 48, 72 or 96 h. Medium and agonists were replaced every 24 h. Medium from each 24 h period of treatment was collected and measured for cortisol, corticosterone and androstenedione content by RIAs. Results shown are mean (\pm s.d.) of triplicate measurements from one of 5 similar experiments for cortisol data and one of 2 experiments for corticosterone and androstenedione data. * $P < 0.05$, *** $P < 0.01$ compared with basal secretion.

CORTISOL response to chronic AII / forskolin treatment							
Exposure time of cells to agonist	Response Ratio relative to basal					Mean (n=5)	s.d.
	Expt.1	Expt.2	Expt.3	Expt.4	Expt.5		
All 24 h	3.08	2.23	2.78	2.23	8.33	3.730	2.597
All 48 h	5.53	3.66	4.82	2.51	5.44	4.392	1.290
All 72 h	5.94	4.49	6.11	4.48	8.57	5.919	1.671
All 96 h	5.92	4.79	6.51	3.17	7.87	5.653	1.776
forskolin 24 h	2.97	2.32	3.89	2.89	8.31	4.077	2.432
forskolin 48 h	6.59	4.89	9.93	5.60	11.86	7.773	2.992
forskolin 72 h	10.44	7.97	15.92	5.10	18.76	11.638	5.628
forskolin 96 h	12.98	12.75	24.34	5.42	23.91	15.880	8.120

Table 3.1

Cortisol, corticosterone and androstenedione secretions in response to chronic treatment with AII or forskolin. Cells were treated with growth medium alone (basal) or medium containing AII (10 nM) or forskolin (10 µM) for 24, 48, 72 or 96 h. Medium and agonists were replaced every 24 h. Medium from each 24 h period of treatment was collected and measured for cortisol, corticosterone and androstenedione content by RIAs. Results are presented in these tables as Response Ratios (response to stimulus / basal secretion) for each separate experiment performed (n=5 for cortisol, n=2 for corticosterone and androstenedione) (see next page for corticosterone and androstenedione data)

CORTICOSTERONE response to chronic AII / forskolin treatment					
Response Ratio relative to basal					
Exposure time of cells to agonist	Expt.1	Expt.2	Mean (n=2)	range	
All 24 h	3.90	2.48	3.190	0.710	
All 48 h	6.81	9.61	8.210	1.400	
All 72 h	3.14	10.95	7.045	3.905	
All 96 h	2.04	4.33	3.185	1.145	
forskolin 24 h	6.75	4.67	5.710	1.040	
forskolin 48 h	4.95	8.77	6.860	1.910	
forskolin 72 h	5.58	18.14	11.860	6.280	
forskolin 96 h	5.09	15.49	10.290	5.200	

ANDROSTENEDIONE response to chronic AII / forskolin treatment					
Response Ratio relative to basal					
Exposure time of cells to agonist	Expt.1	Expt.2	Mean (n=2)	range	
All 24 h	0.67	0.77	0.720	0.050	
All 48 h	0.65	0.81	0.730	0.080	
All 72 h	1.10	0.99	1.045	0.055	
All 96 h	0.83	1.03	0.930	0.100	
forskolin 24 h	1.47	1.15	1.310	0.160	
forskolin 48 h	1.10	3.43	2.265	1.165	
forskolin 72 h	1.52	6.77	4.145	2.625	
forskolin 96 h	4.61	8.39	6.500	1.890	

Table 3.1 continued: see previous page for legend.

Chronic treatment of the cells with forskolin also led to a time-dependent increase in steroid responses. Total cortisol output and RR were still increasing after 96 h exposure to forskolin (mean RR = 15.9 (\pm 8.1)-fold, n=5 individual experiments). Corticosterone output and RR were maximal after 72 h forskolin treatment (RRs = 6-fold and 18.1-fold in 2 separate experiments, triplicate incubations performed in each). In contrast to chronic AII treatment, forskolin caused a time-dependent increase in androstenedione secretion and RR, reaching maximums after 72 h and 96 h treatment (RRs = 4.6-fold and 8.4-fold, respectively, from 2 individual experiments, triplicate incubations performed in each) (*Figure 3.2* and *Table 3.1*).

The ratios of secretion rate of one steroid relative to the secretion rate of another steroid (for example, the ratio of corticosterone secretion / cortisol secretion, calculated from the RR for each) are presented in *Table 3.2*.

Both basal levels of aldosterone and levels following treatment of cells with AII or forskolin were generally non-detectable. The lower detection limit for the aldosterone radioimmunoassay used could not be calculated accurately as not enough data was generated to determine precision profiles for each assay. However, aldosterone secretion was measured in 2 separate experiments, in response to AII in one experiment and in response to forskolin in the other (*Table 3.3*).

3.2.2 Steroid responses of cells to acute treatment with angiotensin II or forskolin.

3.2.2.1 Time-course of short-term treatment with AII or forskolin

Cells were treated with growth medium alone (basal) or medium containing AII (10 nM) or forskolin (10 μ M) for 2, 4 or 8 h. *Figure 3.3a*) shows the combined RRs for short-term AII or forskolin treatment from n=3 individual experiments. The cortisol RR was greater at 4 h than at 2 h for both AII (P<0.01 in 2 out of 3 experiments, calculated from triplicate incubations within each experiment) and forskolin (P<0.02 in 2 out of 3 experiments, calculated from triplicate incubations within each experiment). Although the total cortisol outputs at 8 h were greater than

Ratio of RRs					
	Expt.	Chronic AII treatment (hours)			
		24 h	48 h	72 h	96 h
corticosterone / cortisol	1	1.3	1.2	0.5	0.3
	2	1.1	2.6	2.4	0.9
cortisol / androstenedione	1	4.6	8.5	5.4	7.1
	2	3.0	4.5	4.5	4.7
corticosterone / androstenedione	1	5.8	10.5	2.9	2.5
	2	3.2	11.9	11.1	4.2
	Expt.	Chronic forskolin treatment (hours)			
		24 h	48 h	72 h	96 h
corticosterone / cortisol	1	2.3	0.8	0.5	0.4
	2	2.0	1.8	2.3	1.2
cortisol / androstenedione	1	2.0	6.0	6.9	2.8
	2	2.0	1.4	1.2	1.5
corticosterone / androstenedione	1	4.6	4.5	3.7	1.1
	2	4.1	2.6	2.7	1.9

Table 3.2

Steroid responses to chronic treatment with AII (10 nM) or forskolin (10 μ M) for 24, 48, 72 or 96 h. Medium and agonists were replaced every 24 h. Medium from the last 24 h of the treatment period was collected and assayed for cortisol, corticosterone and androstenedione content by RIAs. Steroid data was normalised with respect to basal secretion (Response Ratio, RR). These RRs were then expressed themselves as a ratio of secretion of one steroid relative to secretion of a different steroid, for example, corticosterone secretion with respect to cortisol secretion, to give an indication of shift in steroidogenesis from one pathway to another. These ratios are presented in the table above.

ALDOSTERONE response to chronic AII / forskolin treatment			
		Mean (+/- s.d.) (pmol / mg protein / 24 h)	
Exposure time of cells to agonist		Expt. 1	Expt. 2
Basal	24 h	ND	1.85 (0.16)
AII	24 h	ND	4.94 (0.50)
forskolin	24 h	6.37 (1.35)	-
Basal	48 h	ND	1.50 (0.45)
AII	48 h	7.16 (1.23)	5.79 (3.13)
forskolin	48 h	ND	-
Basal	72 h	ND	0.91 (0.09)
AII	72 h	ND	9.06 (2.99)
forskolin	72 h	3.49 (1.85)	-
Basal	96 h	ND	0.97 (0.13)
AII	96 h	ND	13.75 (0.34)
forskolin	96 h	3.00 (1.49)	-

Table 3.3

Aldosterone responses to chronic treatment with AII (10 nM) or forskolin (10 μ M) for 24, 48, 72 or 96 h. Medium and agonists were replaced every 24 h. Medium from each 24 h period of treatment was collected and assayed for aldosterone content. Results presented in the table above are mean (\pm s.d.) from triplicate incubations within each experiment. ND=non-detectable by the RIA used. Cells were not treated with forskolin in Expt.2 . Expt.1 corresponds to Expt.1 in Table 3.1.

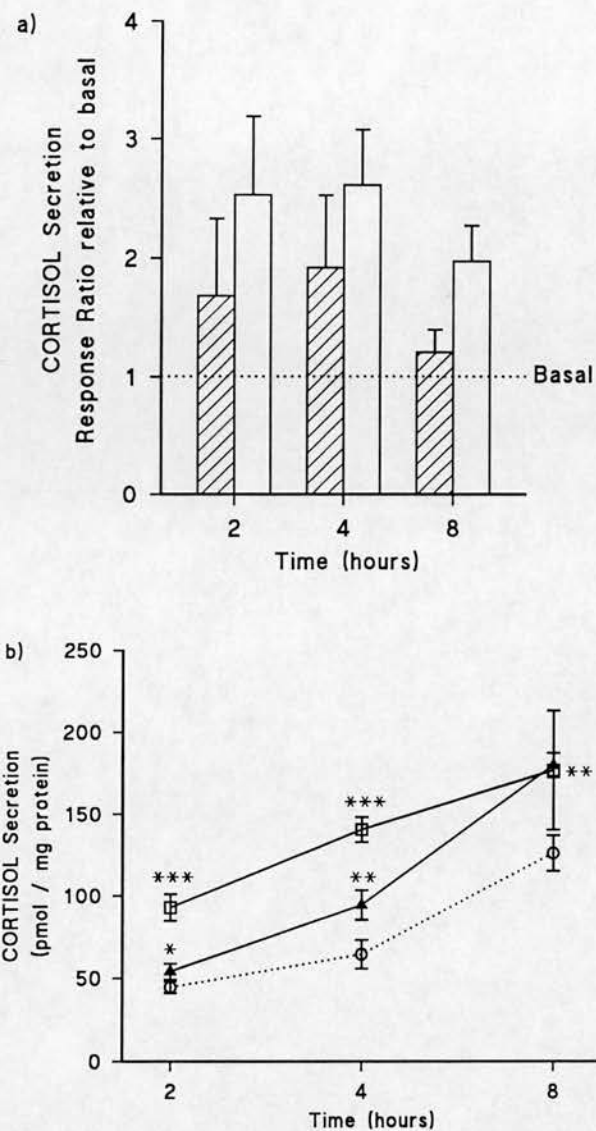


Figure 3.3

Time-course to determine the cortisol response to short-term treatment of H295R cells with AII or forskolin. Cells were treated with growth medium alone (basal) or medium containing AII (10 nM) or forskolin (10 μ M) for 2, 4 or 8 h. After this time, medium was assayed for cortisol content by RIA and data corrected for total cellular protein content. *Figure 3.5a*) shows stimulation of cortisol secretion by AII (hatched bars) or forskolin (open bars), expressed as a Response Ratio (response to stimulus / basal secretion). Results are mean (\pm s.d.) of combined data from n=3 experiments, triplicate incubations performed in each. *Figure 3.5b*) shows a representative experiment (from the set of n=3) demonstrating that the medium concentration of cortisol continues to increase at 8 h, with respect to 4 h, even though the Response Ratio does not. Results are mean (\pm s.d.) of triplicate measurements. (Basal O, AII ▲, forskolin □). *P<0.05, **P<0.02, ***P<0.01 compared with basal secretion.

after 4 h agonist exposure (*Figure 3.3b*)), the RRs were less at 8 h than after 4 h for both AII and forskolin treatments (*Figure 3.3a*)).

3.2.2.2 Steroid responses of cells to 4 h treatment with AII or forskolin

Cells were treated with growth medium alone (basal) or medium containing AII (10 nM) or forskolin (10 μ M) for 4 h (selected on the basis of the time-courses performed, see section 3.2.2.1 above) at 37°C. *Figure 3.4* shows the combined cortisol, corticosterone and androstenedione responses (expressed as mean Response Ratios \pm s.d.) obtained from n=4 individual experiments for cortisol and corticosterone and n=3 individual experiments for androstenedione. Data from the individual experiments are presented in *Table 3.4*, expressed as pmol / mg cellular protein / 4 h. Forskolin significantly stimulated cortisol secretion (2.1 (\pm 0.6)-fold increase over basal for n=4 separate experiments. $P < 0.05$ in 4/4 experiments, $P < 0.01$ for 3 of these 4, calculated from triplicate incubations within each experiment) and corticosterone secretion (2.4 (\pm 0.6)-fold increase over basal for n=4 experiments. $P < 0.01$ in 3/4 experiments, calculated from triplicate incubations within each experiment). Forskolin only stimulated androstenedione secretion over basal levels in 1/3 experiments ($P < 0.05$, calculated from triplicate incubations within the experiment) (*Table 3.4*).

The steroid response to 4 h AII treatment was variable: AII stimulated cortisol and corticosterone secretion in 2/4 experiments ($P < 0.01$ for 2/4 expts for both cortisol and corticosterone responses, calculated from triplicate incubations within each experiment), failing to stimulate either steroid in the other 2 experiments ($P \geq 0.05$) (*Table 3.4*). For the 2 experiments where AII treatment elicited a steroid response, the RRs were 2.3-fold and 2.3-fold for cortisol and 2.6-fold and 2.8-fold for corticosterone. 4 h AII treatment did not alter androstenedione secretion above basal levels ($P \geq 0.05$ in 3/3 experiments, calculated from triplicate incubations within each experiment).

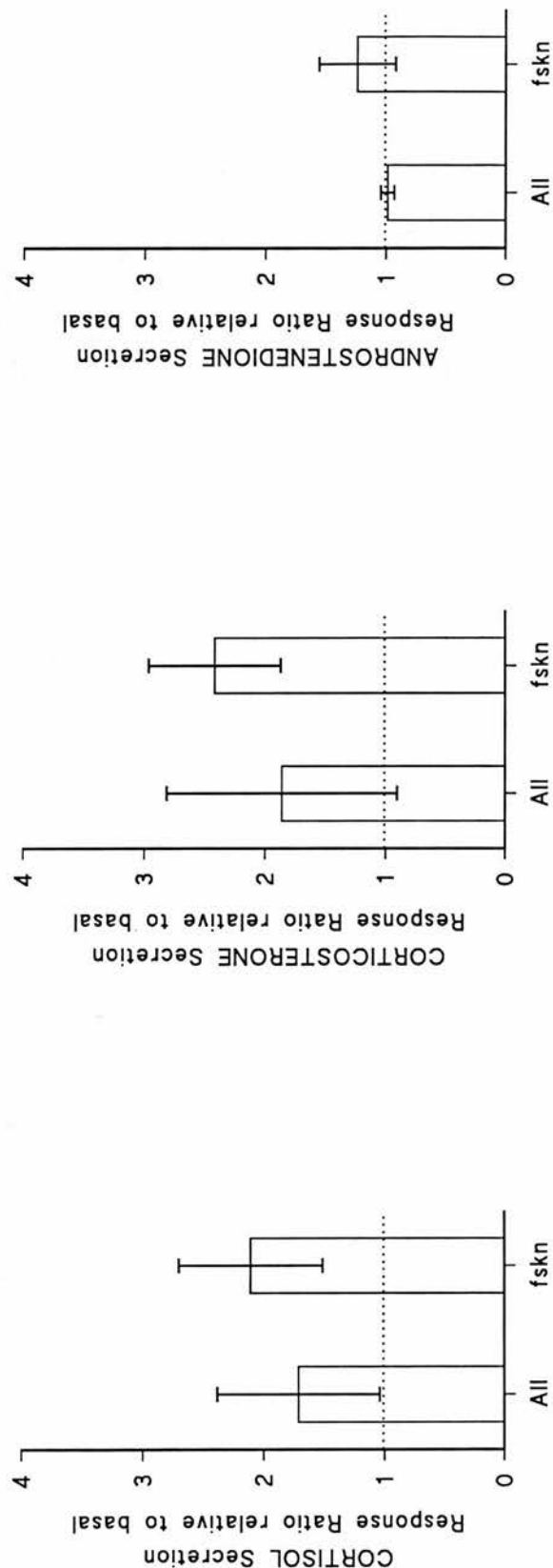


Figure 3.4

Effects of short-term (4 h) treatment with AII or forskolin on cortisol, corticosterone and androstenedione secretion from H295R cells. Cells were treated for 4 h with growth medium alone (basal, dotted line) or medium containing AII (10 nM) or forskolin (10 μ M). Medium contents of the 3 steroids were determined by RIAs, corrected for total cellular protein. Results presented here are mean (\pm s.d.) of combined data from n=4 experiments for cortisol and corticosterone and n=3 experiments for androstenedione, expressed as Response Ratios relative to basal secretion.

CORTISOL Response to 4 h All / forskolin Treatment				
	Mean (+/- s.d.) CORTISOL secretion (pmol / mg protein / 4 h)			
	Expt.1	Expt.2	Expt.3	Expt.4
basal	23.1 (4.9)	104.7 (13.3)	169.1 (52.6)	21.9 (1.8)
All	52.4 (3.1)***	121.7 (5.7)	184.7 (3.2)	50.6 (2.2)***
forskolin	68.1 (2.5)***	183.0 (27.8)***	276.5 (71.3)*	45.6 (1.1)***

CORTICOSTERONE Response to 4 h All / forskolin Treatment				
	Mean (+/- s.d.) CORTICOSTERONE secretion (pmol / mg protein / 4 h)			
	Expt.1	Expt.2	Expt.3	Expt.4
basal	2.3 (0.2)	19.6 (2.7)	12.8 (2.7)	6.7 (1.1)
All	6.0 (1.1)***	19.5 (3.7)	13.6 (4.2)	19.0 (1.6)***
forskolin	6.9 (0.9)***	44.5 (3.4)***	21.9 (7.0)	18.6 (1.8)***

ANDROSTENEDIONE Response to 4 h All / forskolin Treatment				
	Mean (+/- s.d.) ANDROSTENEDIONE secretion (pmol / mg protein / 4 h)			
	Expt.1	Expt.2	Expt.3	
basal	59.6 (19.2)	42.5 (7.8)	25.0 (6.2)	
All	62.6 (1.9)	40.1 (4.2)	24.3 (5.6)	
forskolin	62.6 (1.7)	45.1 (6.8)	40.2 (9.2)*	

Table 3.4

*Cortisol, corticosterone and androstenedione secretions in response to 4 h treatment with growth medium alone (basal), All (10 nM) or forskolin (10 μ M). Cortisol, corticosterone and androstenedione contents of 4 h media were measured by RIAs and corrected per mg cellular protein. Results presented are mean (\pm s.d.) for triplicate incubations for each of n=4 experiments for cortisol and corticosterone and n=3 experiments for androstenedione. Statistically significant changes in secretion compared with basal secretion are indicated by *P<0.05 and ***P<0.01.*

3.2.3 Effects of 96 h pretreatment with AII or forskolin on subsequent steroid responses to 4 h agonist treatment

3.2.3.1 Effect of different pretreatment times on subsequent agonist-induced steroid response.

A preliminary experiment was performed to determine the optimum time-period for pretreating the cells with AII or forskolin, prior to 4 h agonist treatment. Cells were pretreated with AII (10 nM) or forskolin (10 μ M) for 24, 48, 72 or 96 h. Medium and agonists were replaced every 24 h. Following the defined pretreatment period, cells were washed and treated for a further 4 h with fresh growth medium alone (basal) or medium containing AII (10 nM) or forskolin (10 μ M). *Figure 3.5* shows cortisol, corticosterone and androstenedione responses to 4 h treatment with the above agonists following different periods of pretreatment with AII or forskolin ("control" cells were maintained in exactly the same way as pretreated cells, except there was no pretreatment agonist added to the medium).

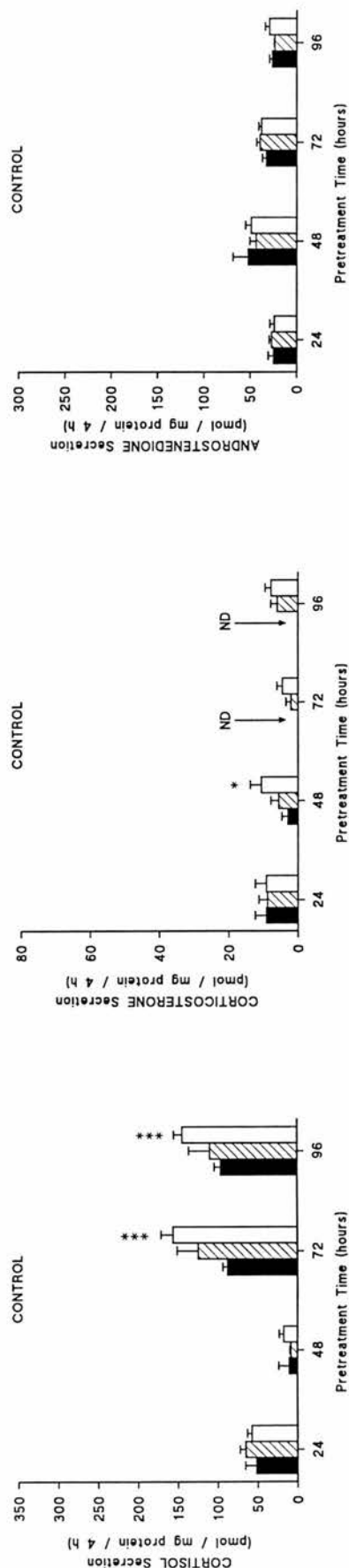
The cortisol and corticosterone responses of the cells to 4 h AII or 4 h forskolin were increased following pretreatment with either AII or forskolin, reaching a plateau after 72-96 h pretreatment with either AII or forskolin. Androstenedione secretion in response to 4 h AII or 4 h forskolin was unaltered, relative to basal levels, from AII pretreated H295R cells. Forskolin pretreatment, in contrast, increased the subsequent androstenedione responses to 4 h AII and 4 h forskolin, with the 4 h forskolin response reaching a plateau after 48 h pretreatment and the 4 h AII response still increasing after 72-96 h pretreatment (*Figure 3.5*). A 96 h pretreatment time was chosen for further experiments.

3.2.3.2 Alteration of the steroidogenic phenotype of H295R cells following pretreatment with angiotensin II or forskolin

96 h treatment with AII (10 nM) caused no change in 4 h basal androstenedione production ($P \geq 0.05$ in 4/4 individual experiments, calculated from triplicate incubations within each experiment) but significantly increased basal

Figure 3.5

Time-course to determine length of time to pretreat H295R cells with either AII or forskolin to optimise the responsiveness of the cells prior to 4 h treatment with agonists. Cells were pretreated with growth medium alone (control) or medium containing AII (10 nM, AII pretreatment) or forskolin (10 μ M, forskolin pretreatment) for 24, 48, 72 or 96 h. After this time, cells were washed thoroughly and then treated for 4 h with fresh growth medium alone (basal, solid bars) or medium containing AII (10 nM, hatched bars) or forskolin (10 μ M, open bars). After 4 h, medium was collected and assayed for cortisol, corticosterone and androstenedione content by RIAs and data was corrected per mg cellular protein. Results are presented as mean (\pm s.d.) from triplicate incubations from one experiment. Statistically significant secretion compared with basal secretion is indicated by * P <0.05 and *** P <0.01. ND=non-detectable in the assay used. See next page for “AII pretreatment” and “Forskolin pretreatment”



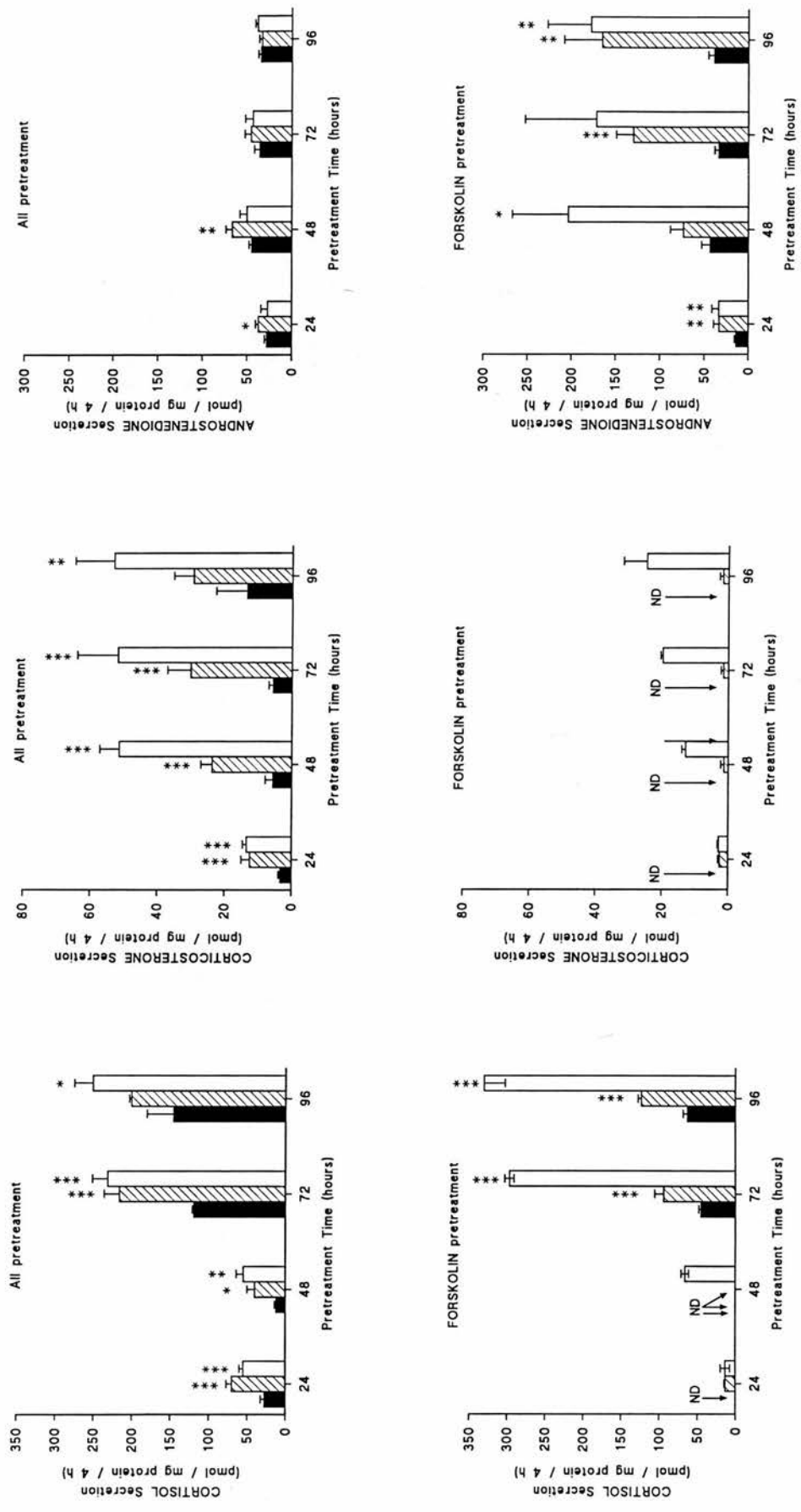


Figure 3.5 continued: see previous page for figure legend.

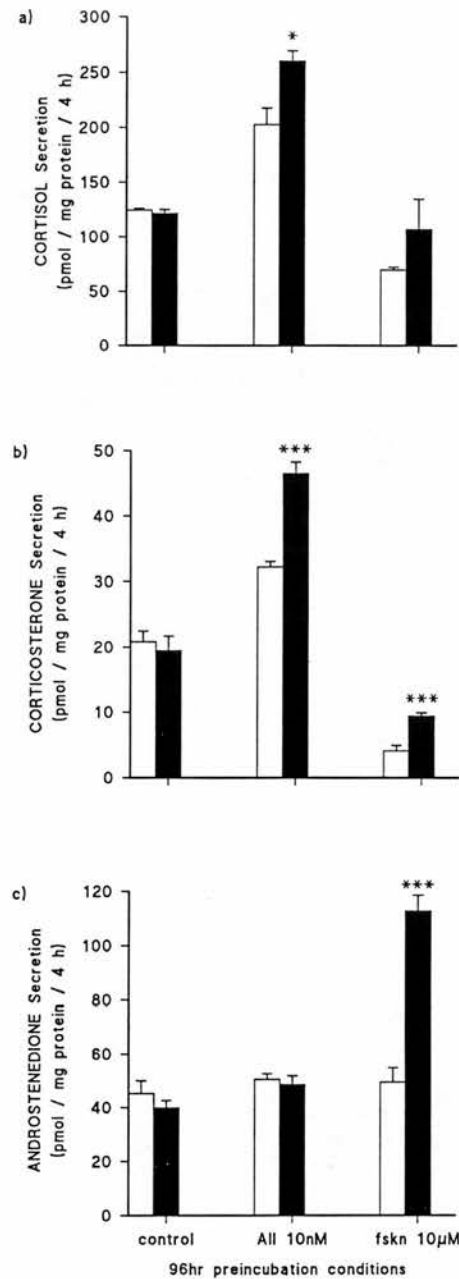


Figure 3.6

Different profiles of a) cortisol, b) corticosterone and c) androstenedione responses to 4 h AII treatment following 96 h pretreatment under different conditions. Cells were preincubated for 96 h with growth medium alone (control) or medium containing AII (10 nM) or forskolin (10 μM). After washing, cells were subsequently treated for 4 h with or without AII (10 nM). These graphs show 4 h basal (open bars, □) and 4 h AII stimulated (solid bars, ■) secretions of the 3 steroids following the different preincubation conditions. Results are expressed as mean (±s.d.) for triplicate incubations from a representative experiment of a set of n=4 experiments. *P<0.05, ***P<0.01 compared with basal secretion.

Ratio of RRs (mean +/- s.d.)			
	96 h All preincubation	96 h fskn preincubation	Statistics
corticosterone/androstenedione	1.6 (+/- 0.07)	1.1 (+/- 0.24)	P<0.05
cortisol/androstenedione	1.3 (+/- 0.08)	1.1 (+/- 0.45)	NS
cortisol/corticosterone	0.8 (+/- 0.06)	1.1 (+/-0.57)	NS

Table 3.5

Steroid responses to 4 h treatment with AII (10 nM) following 96 h preincubation of the cells with growth medium alone (control) or containing AII (10 nM) or forskolin (fskn, 10 μ M). Medium from the 4 h treatment period was collected and assayed for cortisol, corticosterone and androstenedione content. Data obtained by RIA for each steroid were normalised with respect to basal secretion (Response Ratio). These ratios were then expressed themselves as a ratio of secretion of one steroid with respect to secretion of a different steroid, for example, corticosterone secretion with respect to androstenedione secretion (corticosterone / androstenedione), to give an indication of shift in steroidogenesis from one pathway to another induced by the different pretreatment conditions. These ratios are presented in the table above. Statistically different ratios between the different pretreatment conditions are taken at P<0.05. Values of P \geq 0.05 were considered non-significant (NS).

cortisol secretion ($P < 0.05$ in 4/4 experiments, calculated from triplicate incubations within each experiment) and basal corticosterone secretion ($P < 0.05$ in 3/4 experiments, calculated from triplicate incubations within each experiment). In contrast, 96 h forskolin pretreatment significantly decreased 4 h basal production of all three steroids ($P < 0.05$ in 3/4 experiments, calculated from triplicate incubations within each experiment).

4 h treatment of H295R cells with AII (10 nM) following 96 h preincubation with AII resulted in a RR of 1.3 (± 0.2)-fold for cortisol, 1.7 (± 0.4)-fold for corticosterone and 0.9 (± 0.1)-fold for androstenedione (n=4 experiments for all three steroids). This compared with RRs of 1.5 (± 0.2)-fold, 1.6 (± 0.5)-fold and 1.7 (± 0.6)-fold for cortisol, corticosterone and androstenedione respectively after 96 h preincubation with forskolin (n=4 experiments for all three steroids) (*Figure 3.6*). If the RRs for corticosterone and androstenedione are expressed as a ratio of increased corticosterone secretion to androstenedione secretion and these ratios are taken as an indication of shift in steroid production away from adrenal androgens and towards 17-deoxysteroid secretion or vice versa, then following AII preincubation the ratio was 1.6 (± 0.07) whereas following forskolin preincubation the ratio was 1.1 (0.24) (n=4). Statistical comparison of these ratios was significant with $P < 0.02$ (*Table 3.5*).

3.3 Discussion

Most previous studies which have investigated the steroid responses to agonists from H295R cells have either looked at one steroid in particular or, where several steroids have been measured, have only examined secretion following a fixed period of incubation with each agonist, usually 48 h (Bird *et al*, 1995b, 1996a, 1996b; Mason *et al*, 1995; Rainey *et al*, 1993). In this chapter, the time-dependent changes in chronic secretion of cortisol, corticosterone, androstenedione (and aldosterone) to AII or forskolin were examined, along with the short-term responses to these agonists. Aldosterone, cortisol and androstenedione were measured because they are end-pathway steroids produced in the adrenal (*Figure 1.3*). However, due to the difficulties experienced with the detection of aldosterone secretion, corticosterone production was measured instead. The ability to alter the steroidogenic phenotype of the cell-line was investigated.

The profiles for cortisol, corticosterone and androstenedione secretion following increasing lengths of chronic treatment with AII or the protein kinase A activator, forskolin, were investigated (*Figure 3.2*). Forskolin was used to mimic the ACTH-induced activation of protein kinase A, since H295R cells show poor response to ACTH (Rainey *et al*, 1993), which could relate to the low levels of ACTH receptor mRNA found in these cells (Mountjoy *et al*, 1994). Preliminary experiments using ACTH were also performed in this study, which confirmed that ACTH was a poor agonist for cortisol, corticosterone and androstenedione secretion from control cells and, although the steroid responses to ACTH increased following pretreatment of the H295R cells with AII or forskolin, the steroid responses were still poor compared to those elicited by forskolin under the same conditions (*data not shown*).

The steroid secretion profiles obtained for chronic treatment were different for AII and forskolin. By examining the ratios of corticosterone secretion to cortisol secretion (that is, corticosterone / cortisol) and corticosterone / androstenedione (*Table 3.2*), it is clear that 48 h exposure of the cells to AII predominantly stimulates corticosterone secretion in these cells. In contrast, by 96 h exposure to AII there was

a relative decrease in the ratios of corticosterone / cortisol and corticosterone / androstenedione, indicating a shift away from corticosterone production. This could be the result of a relative increase in cortisol secretion, a relative decrease in corticosterone secretion or both. The fact that androstenedione secretion did not increase at 96 h suggests that corticosterone production must have decreased to some extent. Indeed, the RR for corticosterone production to AII fell (35 % in 1 experiment and 60 % in the other experiment) after 96 h treatment, whereas the cortisol RR remained constant. Thus, AII appears to predominantly stimulate the mineralocorticoid pathway up to 48 h treatment, but further exposure leads to a decrease in this effect. AII markedly increases 3 β HSD mRNA and activity in H295R cells whilst having a lesser effect on P450c17 mRNA expression and activity (Rainey *et al*, 1994; Bird *et al*, 1996b). These effects were observed after 20 / 24 h treatment with AII (Rainey *et al*, 1994; Bird *et al*, 1996b), which would be in keeping with the steroid responses seen after 48 h in this study. The pattern of 3 β HSD and P450c17 mRNA expression in cultured human zfr cells in response to AII shows maximal increase in 3 β HSD expression at 24 h, gradually decreasing at 48, 72 and 96 h, though still remaining elevated with respect to 12 h-treated cells. In the same study, P450c17 mRNA levels remained constant up to 96 h (Lebrethon *et al*, 1994). Thus, a decrease in 3 β HSD mRNA at 96 h, coupled with an unaltered level of P450c17 mRNA, would result in a decrease in the ratio of 3 β HSD / P450c17 transcripts at 96h with respect to 48 h. If the assumption is made that the altered transcript ratio is reflected in a corresponding change in enzyme synthesis (and activity) (Hum & Miller, 1993; Parker & Schimmer, 1993), the outcome would be a decrease in corticosterone secretion relative to cortisol and androstenedione secretion. A similar effect in H295R cells could explain the change in steroid responses to AII seen with increasing length of treatment.

A further possibility is that the cells may have become desensitised to AII. Sensitivity of cells to AII can decrease in a number of ways; the surface AII receptor numbers can decrease by internalisation (Ouali *et al*, 1997) or the phosphoinositide response may diminish following prolonged exposure to AII (Enyedi & Spat, 1987). However, since the cortisol response did not decline at 96 h (compared with the

corticosterone response), this explanation appears less plausible. Indeed, whilst chronic AII treatment has been shown to cause an initial decrease in AT₁-receptor mRNA from H295R cells, little effect on AII-binding or subsequent phosphoinositidase C response was observed (Bird *et al*, 1995a).

With increasing length of chronic forskolin treatment, the ratios of corticosterone / cortisol and corticosterone / androstenedione progressively decreased from 24 h to 96 h, consistent with a shift away from corticosterone production towards cortisol and androstenedione production. Rainey *et al* (1994) demonstrated that 48 h treatment with activators of the protein kinase A pathway (forskolin and dbcAMP) greatly increase the expression and activity of P450c17, accompanied by an increase in cortisol and androstenedione secretion. It can be seen in *Figure 3.2* that cortisol and androstenedione productions are still increasing after 72-96 h forskolin treatment. This would suggest that the inhibitory mechanisms regulating the chronic AII response after 72-96 h are different from those controlling chronic forskolin action. The steroid secretory response over the period 72-96 h exposure to forskolin was greater than that seen over the same period in response to AII. This again demonstrates the different regulatory mechanisms involved in the control of chronic AII or forskolin response.

The stimulation of corticosterone by forskolin probably reflects an overall increase in steroidogenesis by the cells, since forskolin has been shown to increase expression of several steroidogenic enzymes to varying degrees, including P450scc, 3 β HSD, P450c17 and P450c21 (Rainey *et al*, 1993; Bird *et al*, 1995b; Staels *et al*, 1993).

An unexpected finding was that basal aldosterone levels were rarely detected and neither AII nor forskolin consistently gave a detectable increase in aldosterone secretion. Bird *et al* (1993, 1996b) characterised an aldosterone response from H295R cells to both AII and forskolin. Moreover, both AII and forskolin increase aldosterone synthase mRNA levels, the greater effect being produced by AII (Bird *et al*, 1993; Holland *et al*, 1993). However, basal levels of P450aldo mRNA and basal secretion of aldosterone in these cells were reportedly low (Holland *et al*, 1993). In the studies reported here, basal aldosterone was detected in only 1 experiment and

stimulated aldosterone in only 2 experiments (in response to AII in one and to forskolin in the other) (see *Table 3.3*). The loss of a measurable aldosterone response, either basal or stimulated, suggests an extremely low level of aldosterone synthase activity. Since the corticosterone response was readily measurable, this would further support this explanation. Slight differences in culture conditions, compared with other studies in these cells could affect P450_{aldo} expression or activity, and may explain the differences in ability to measure aldosterone. Another possibility is that the cells have mutated and lost their ability to express this enzyme, though this is less likely given the long period of establishment of the cell-line (approximately 7 years) (Gazdar *et al*, 1990) and the maintenance of an extensive steroidogenic enzyme complement after this time (Staels *et al*, 1993). Moreover, aldosterone secretion was measurable on 2 separate occasions.

The short-term (4 h) steroid responses to AII and forskolin were also examined in this cell-line. A 4 h incubation was chosen for these short-term treatment studies because the RR was higher than at 8 h and the total steroid output was higher than at 2 h, aiding measurement of the steroids by RIA (*Figure 3.3*).

Forskolin stimulated an increase in cortisol and corticosterone secretions but failed to significantly increase androstenedione secretion above basal. This contrasts with the chronic action of forskolin, which increased androstenedione secretion in a time-dependent manner (*Figure 3.4*).

The cortisol and corticosterone responses to AII were inconsistent from one experiment to another. In 2/4 experiments, AII stimulated a significant increase in cortisol and corticosterone ($P < 0.01$ for both steroids) (*Table 3.4*) but failed to do so in the other 2 experiments ($P \geq 0.05$). As with chronic treatment, AII had no effect on androstenedione secretion (*Figure 3.4*).

The responsiveness (in terms of RR) of these cells to 4 h treatment differed from that observed with chronic (24-96 h) treatment. The importance of ACTH in the maintenance of steroidogenic capacity of cells was first demonstrated in rat adrenal cortex, where it was discovered that hypophysectomy led to a rapid fall in both microsomal and mitochondrial cytochromes P450 (Kimura *et al*, 1969; Pfeiffer *et al*, 1972; Purvis *et al*, 1973). ACTH administration restored enzyme levels.

Cultured adrenal cells from several species quickly lose their ability to synthesise steroids. For example, human adrenal zfr cells in culture exhibit a rapid and progressive decline in levels of P450_{scc}, P450_{17 α} and 3 β HSD mRNAs and proteins, which were restored, to varying degrees, upon treatment with ACTH or AII (Lebrethon *et al*, 1994). If recently isolated and cultured cells can rapidly lose their steroidogenic capacity, it is not inconceivable that the basal steroidogenic enzyme levels of the long-standing H295R cell-line may be very low. This would explain the poorer responsiveness of the cells to short-term stimulation.

It was therefore decided to 'prime' the cells before investigating the steroid responses to 4 h stimulation. The reasons for this were two-fold: 1) to increase the steroidogenic capacity of the cells prior to acute treatment and 2) to investigate whether it was possible to alter the steroidogenic phenotype of the cell-line, depending on the choice of pretreatment agonist. Based on known effects on steroid enzyme expression, it was expected that pretreatment of the cells with AII would confer a glomerulosa-like phenotype on the cells whereas pretreatment with forskolin (to mimic ACTH-induced protein kinase A activation) would confer a more fasciculata-reticularis phenotype on the cells.

Previous studies on the chronic effects of AII or forskolin on these cells suggested that steroidogenic responsiveness was maximal with 72 h or 96 h treatment (*Figure 3.2*). The pretreatment time-course (*Figure 3.5*) demonstrates that the responsiveness to acute agonist treatment is also highest after 72 h or 96 h pretreatment with AII or forskolin. A pretreatment time-length was chosen that was close to optimal for all 3 steroids being measured. For AII and forskolin pretreatments, there was little difference between 72 h or 96 h pretreatment on the subsequent AII-induced cortisol, corticosterone or androstenedione responses. Since the AII-induced androstenedione response was possibly marginally better following 96 h pretreatment, this time period was chosen for further experiments.

Both AII and forskolin pretreatment increased the responsiveness (RR) of the cells to a subsequent 4 h AII exposure (*Figure 3.6*). Indeed, AII had little effect on steroid secretion from "control" cells, but significantly stimulated steroid secretion after "AII pretreatment" or "forskolin pretreatment".

The profiles of steroids secreted differed depending on the pretreatment agonist. A 96 h pretreatment with AII resulted in an increase in AII-stimulated cortisol and corticosterone ($P < 0.05$ and $P < 0.01$, respectively, $n = 4$), whereas significant increases in androstenedione and corticosterone secretions were seen after forskolin pretreatment ($P < 0.01$ for both steroids, $n = 4$) (*Figure 3.6*). If the ratio of increased corticosterone secretion to androstenedione secretion is calculated and used as a measure of the shift in steroid production away from adrenal androgens and towards 17-deoxysteroid secretion or vice versa (*Table 3.4*), it is clear that H295R cells produce more androstenedione in relation to corticosterone following forskolin preincubation as compared to AII preincubation. Thus, chronic forskolin treatment alters steroid production towards the adrenal androgen pathway and away from 17-deoxysteroid production, favouring a more zfr-like phenotype. This observation is consistent with what is already known in relation to the effects of forskolin and AII on expression of steroid pathway enzymes. Thus, the ratio of 3β HSD / P450c17 mRNA most probably increases following AII treatment (favouring the mineralocorticoid pathway) and decreases following forskolin treatment (favouring the glucocorticoid / androgen pathways).

Interestingly, basal cortisol and corticosterone secretions were lower following 96 h forskolin pretreatment, compared with “control” cells. In contrast, AII pretreatment increased basal secretion of cortisol and corticosterone. Basal androstenedione secretion was unaffected by either pretreatment agonist. The reason for these changes in basal steroid secretion is unclear. As mentioned before, chronic forskolin treatment raises levels of P450c17 mRNA and activity, resulting in a decrease of the 3β HSD / P450c17 ratio, and a shift in steroidogenesis towards adrenal androgens. If the lyase activity of P450c17 was induced to a greater degree than the hydroxylase activity, pregnenolone would be converted mainly to DHEA. Consequently, pregnenolone and 17-OH-pregnenolone pools may become smaller. The decrease in starting substrate for the mineralocorticoid and glucocorticoid pathways could result in a decrease in basal corticosterone and basal cortisol secretion. Basal androstenedione levels would be unaffected if the DHEA pool was already large, such that an increase in 17,20-lyase activity and DHEA formation did

not actually increase the size of this pool to any significant degree. The initial characterisation of these cells by Gazdar *et al* (1990) suggests that DHEA levels in these cells are high. Mechanisms controlling the differential regulation of 17 α -hydroxylase activity and 17,20-lyase activity of P450c17 are still unclear. It has been suggested that the amount of available P450 oxidoreductase may determine the ratio of one activity to another (Yanagibashi & Hall, 1986). Whilst this may explain the differences in 17 α -hydroxylase and 17,20-lyase activities between the testes and the adrenal (the molar ratio of P450 oxidoreductase to P450c17 is 3-fold higher in the testes than the adrenal), it does not easily explain changes in 17 α -hydroxylase and 17,20-lyase activities within the adrenal gland itself. Miller *et al* (1997) recently supported these findings and added that post-translational phosphorylation of the P450c17 protein appears to increase the efficiency of electron transfer between P450c17 and P450 oxidoreductase. Post-translational phosphorylation of P450c17 seems to be cAMP-dependent (Miller *et al*, 1997). Another possibility for the intra-adrenal regulation of 17 α -hydroxylase and 17,20-lyase activities stems from the findings of Couch *et al* (1986), who noted that various adrenal steroids appeared to inhibit the 17 α -hydroxylase reaction to a greater extent than the 17,20-lyase reaction.

Chronic AII pretreatment, on the other hand, increases expression of 3 β HSD, resulting in a relative increase in the 3 β HSD / P450c17 ratio, compared with “control” cells. This would result in conversion of pregnenolone primarily to progesterone and 17 α -OH-pregnenolone to 17 α -OH-progesterone, with relatively little conversion to DHEA. Again, the change in the size of the DHEA pool may be negligible, so that an increase in basal androstenedione is not seen. Channeling of steroidogenesis down the mineralocorticoid and glucocorticoid pathways could explain the increase in basal corticosterone and cortisol seen following chronic AII pretreatment. Subsequent acute AII treatment would increase steroidogenesis primarily by increasing cholesterol transfer to P450scc (involving AII-stimulated upregulation of StAR expression (Clark *et al*, 1995)), rather than further altering the enzyme ratios. Thus the ratios of enzymes induced by the pretreatment conditions would determine the pattern of steroids seen in response to acute exposure to AII.

Current theories suggest that cells in the three zones of the adrenal cortex arise from a common undifferentiated, progenitor cell because transposition of cells from all zones of the adrenal cortex to *in vitro* culture results in a loss of their zone-specific properties (Hornsby & Crivello, 1983) (section 1.3.1). Migration of a cell from its origin in an outer stem cell layer through all three zones of the cortex has been demonstrated by the appearance of a pattern of radial stripes of a 21-hydroxylase promoter / β -galactosidase reporter transgene throughout the cortex of transgenic mice (Morley *et al*, 1996). Precisely what determines and regulates the differentiated phenotype of this cell is still unclear. The ability of the H295R cell-line's steroid phenotype to be shifted from predominantly one pathway to another means that this cell-line could be a useful model for studying mechanisms regulating zonation and the differential expression of the steroid pathways. Moreover, these cells could potentially be used to estimate the effects of different putative steroidogenic agonists on zg-like function or zfr-like function.

4.0 Steroid responses of H295R cells to short-term and chronic treatment with VIP

4.1 Introduction

There is now strong evidence for innervation of the adrenal cortex, raising the question of a neuroendocrine regulation of steroidogenesis (section 1.6.4.1). In addition, the intermingling of adrenocortical and medullary chromaffin tissues (section 1.6.4.2) begs the question as to whether chromaffin cell secretagogues may also modulate adrenocortical steroid secretion in a paracrine fashion.

So far, most studies into the paracrine and neuroendocrine control of steroidogenesis have been limited to species other than the human. Because many of these potential agonists have established indirect effects on steroidogenesis, for example, via actions on the HPA axis, it has sometimes been difficult to distinguish between indirect and direct actions *in vivo*. The presence of a paracrine / neuroendocrine mechanism of regulation can be investigated with the aid of *in vitro* studies. However, the difficulty in obtaining fresh normal, human adrenal glands post-mortem or at operation has hindered investigations on cultured human adrenocortical cells.

VIP has been shown to influence adrenal function in several ways, including effects on growth (Rebuffat *et al*, 1994) and adrenal blood flow (Bloom *et al*, 1987). In addition, a function for VIP in the circadian rhythm has been suggested (Cugini *et al*, 1991). In this chapter, the H295R human adrenocortical tumour cell-line was used to investigate a direct action of VIP on adrenocortical steroidogenesis.

4.1.1 Regulation of adrenal steroidogenesis by VIP

VIP has been shown to stimulate steroid secretion from adrenocortical cells. Precisely how VIP mediates this effect is still unclear. Plenty of evidence exists for an indirect modulation of steroidogenesis by VIP. In addition to increasing perfusion flow rate through isolated, perfused rat adrenals (Hinson *et al*, 1994a), VIP also

decreased vascular resistance in conscious, functionally hypophysectomised calves (Bloom *et al*, 1987). Thus, VIP may increase blood flow through the adrenal *in vivo*, thereby leading to an increase in steroid secretion (section 1.6.3). Within the adrenal gland, stimulation of the splanchnic nerves results in vasodilation of adrenal blood vessels and VIP has been postulated to be a neurotransmitter released following splanchnic nerve activation (Bloom *et al*, 1988).

VIP has also been shown to stimulate the release of catecholamines from medullary chromaffin tissue in rats and dogs (Wakade *et al*, 1991; Yamaguchi, 1993). The steroidogenic effect of VIP has been attributed by many authors to an indirect action requiring VIP-stimulated catecholamine release from medullary chromaffin cells (also found within the adrenal cortex (section 1.6.4.2)) and subsequent stimulation of steroidogenesis by the action of catecholamines on adrenocortical cells. Evidence for this indirect action includes the observation that VIP-stimulated aldosterone and corticosterone secretion from rat adrenals was attenuated by β -adrenoceptor antagonists (Bernet *et al*, 1994a; Hinson *et al*, 1992; Mazzocchi *et al*, 1993a). Recently, Bornstein *et al* (1996) reported steroid secretion in response to VIP from dispersed human adrenal cells, which was attenuated by a β -adrenoceptor antagonist. It was postulated that the steroidogenic effect of VIP was mediated by catecholamine release from interspersed chromaffin cells detected within the culture.

These observations still leave open the question as to whether VIP can stimulate adrenocortical cells directly. Stimulation of steroid secretion in response to VIP was noted from perfused rat and porcine adrenal glands (Hinson *et al*, 1992, 1994b; Ehrhart-Bornstein *et al*, 1991a) and from an intact rat capsule-zg preparation, where the subcapsular neural network remained relatively intact (Hinson *et al*, 1992; Holzwarth *et al*, 1987; Bernet *et al*, 1994a), but not from dispersed rat adrenocortical cells (Hinson *et al*, 1992; Enyedi *et al*, 1983). VIP infusion into conscious calves resulted in increased cortisol production but only in the presence of ACTH infusion (Bloom *et al*, 1987). Mazzocchi *et al* (1994b) advanced the hypothesis that VIP directly stimulated aldosterone and corticosterone secretion from dispersed rat

capsular and inner adrenocortical cells, respectively, by acting on a subset of ACTH receptors.

Further evidence for a direct action of VIP includes the autoradiographic detection of VIP binding sites in rat adrenal cortex (Cunningham & Holzwarth, 1989). These were found throughout the cortex, but were more concentrated in the subcapsular / zg zone. Stimulation of the splanchnic nerves resulted in a release of VIP in the gland, detectable in the adrenal vein of conscious calves (Bloom *et al*, 1987), and also following splanchnic nerve stimulation of isolated, perfused porcine adrenals with an intact nerve supply (Ehrhart-Bornstein *et al*, 1991a). Thus, VIP released within the adrenal may reach concentrations high enough to affect adrenocortical cells in a paracrine manner, by acting on a specific VIP receptor.

The aim of this series of experiments was to investigate the effects of VIP on cortisol, corticosterone and androstenedione production by H295R cells. The effects of chronic (24 - 96 h) and short-term (4 h) treatment with VIP on steroid secretion were investigated. Cells were treated with AII and forskolin as positive controls since the steroid responses of H295R cells to these agonists were characterised in chapter 3.0.

As reported in Chapter 3.0, the steroidogenic capacity of H295R cells can be increased by pretreatment with either angiotensin II or forskolin. Moreover, depending on the pretreatment agonist used, the steroidogenic phenotype of the cells can be altered. Chronic pretreatment of cells with AII resulted in a potentially zona glomerulosa-like (zg-like) phenotype of the cells, whereas forskolin pretreatment induced a more fasciculata / reticularis-like (zfr-like) steroidogenic phenotype (see Chapter 3.0). The short-term (4 h) steroid responses to VIP following chronic (96 h) pretreatment of the cells with either AII or forskolin were investigated, and compared with “control” cells (no agonist added to medium during pretreatment period), based on the hypotheses: 1) the increase in steroidogenic capacity induced by pretreatment might increase the magnitude of the steroid responses to VIP and 2) VIP may act preferentially on a particular zone of the adrenal cortex. By pretreating the cells with

AI1 or forskolin, the actions of these agonists on zg-like and zfr-like steroid phenotypes could be examined.

The effect of 96 h pretreatment of H295R cells with VIP itself on the subsequent 4 h VIP-induced cortisol response was also investigated.

Finally, a pretreatment time-course was performed to determine the optimum length of time to pretreat the cells with forskolin to optimally enhance the 4 h VIP-induced cortisol response.

4.2 Results

4.2.1 Steroid responses from H295R cells following 24 h treatment with VIP

Table 4.1 shows the cortisol, corticosterone and androstenedione responses to 24 h treatment with VIP. Cells were also treated with AII (10^{-8} M) and forskolin (10^{-5} M), as positive controls and for comparison of magnitudes of responses.

VIP (10^{-7} M) significantly stimulated cortisol secretion ($2.4 (\pm 2.4)$ -fold, $n=3$ individual experiments, $P<0.01$, calculated from triplicate incubations within each experiment) and corticosterone secretion (2.4 -fold, $P<0.01$, calculated from triplicate incubations in 1 experiment) but failed to stimulate androstenedione secretion above basal levels ($P\geq 0.05$, calculated from triplicate incubations in 1 experiment). Within each experiment, the cortisol response to VIP was less than that seen to either AII or forskolin (see *Table 4.1*).

The steroid responses to increasing incubation times with VIP were investigated. *Figure 4.1* shows the time-course for cortisol, corticosterone and androstenedione secretion in response to 24, 48, 72 and 96 h VIP treatment. The Response Ratios (RR, response to stimulus / basal secretion) for the individual experiments are shown in *Table 4.2*. Although cells were exposed to VIP for the times indicated, medium and agonists were replaced every 24 h and only medium from the last 24 h of each treatment period was collected and assayed for steroid content.

VIP (10^{-7} M) significantly stimulated cortisol and corticosterone secretion after 24, 48, 72 and 96 h treatment ($P<0.01$) (see *Figure 4.1* and *Table 4.2*). The cortisol response to VIP increased with time, causing $2.5 (\pm 1.4)$ -fold, $3.1 (\pm 1.4)$ -fold, $4.5 (\pm 2.6)$ -fold and $6.3 (\pm 4.3)$ -fold increases, relative to basal secretion, after 24, 48, 72 and 96 h treatment, respectively ($n=3$) (see *Figure 4.1*). The data for each individual experiment is presented in *Table 4.2*.

24 h CORTISOL Secretion						24 h CORTICOSTERONE Secretion				24 h ANDROSTENEDIONE Secretion			
		RR (response to stimulus / basal secretion)						RR (response to stimulus / basal secretion)				RR (response to stimulus / basal secretion)	
		Expt. 1	Expt. 2	Expt. 3	Expt. 4			Expt. 1	Expt. 2			Expt. 1	Expt. 2
fskn		*** 2.9	*** 2.3	*** 3.9	*** 8.3	fskn		***3.1	*** 4.7	fskn		1.5	1.2
	All	*** 1.3	*** 2.2	*** 2.8	*** 8.3	All		1	*** 2.5	All		1	*0.77
VIP		*** 1.7	*** 1.6	*** 4.1		VIP			*** 2.4	VIP			1

Table 4.1

Comparison of effects of 24 h treatment with VIP, All and forskolin on cortisol, corticosterone and androstenedione secretion from H295R cells. Cells were treated with growth medium alone (basal) or containing either forskolin (fskn, 10⁻⁵ M), All (10⁻⁸ M) or VIP (10⁻⁷ M) for 24 h. After this time, medium was removed and assayed for cortisol, corticosterone and androstenedione content by separate RIAs. Steroid data was corrected for cellular protein content. Results here are expressed as a RR (response ratio; response to stimulus / basal secretion). Values of 1.0 represent no change with respect to basal secretion and values less than 1.0 represent a decrease in steroid secretion compared with basal. Values greater than 1.0 represent increases in steroid secretion with respect to basal. Statistically significant changes in steroid secretion within any individual experiment are noted by *P<0.05 and ***P<0.01.

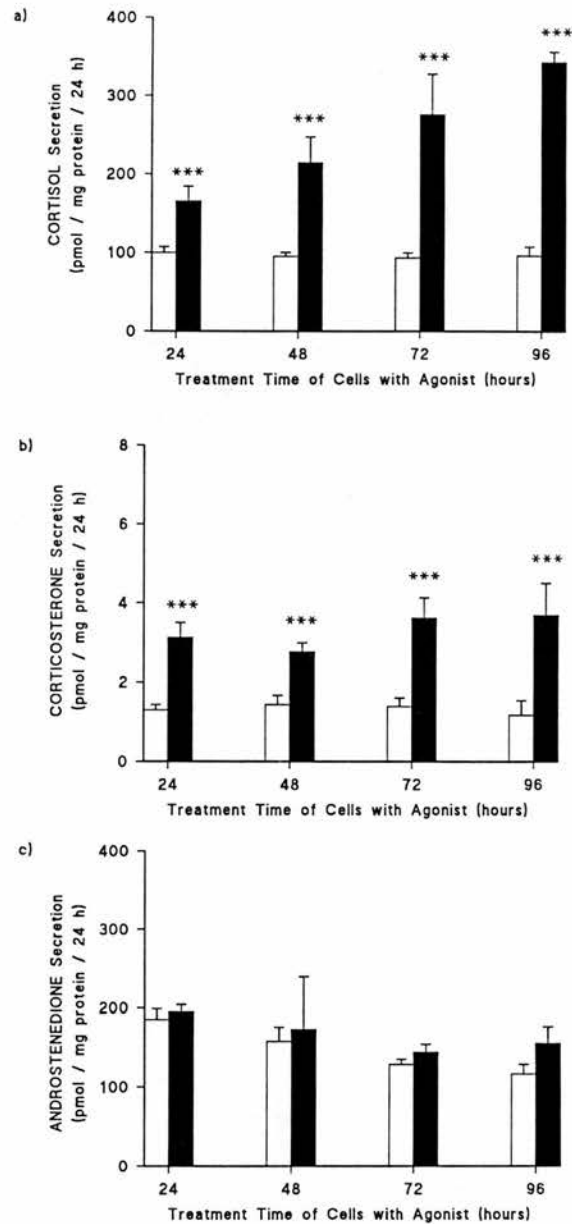


Figure 4.1

Time-dependent changes in a) cortisol, b) corticosterone and c) androstenedione secretions in response to chronic treatment with VIP. Cells were treated with growth medium alone (basal, open bars) or medium containing VIP (10^{-7} M, solid bars) for 24, 48, 72 or 96 h. Medium and agonists were replaced every 24 h. Medium from the last 24 h of each treatment period was collected and assayed for steroid content by separate RIAs. Steroid data was corrected for total cellular protein. Results shown are mean (\pm s.d.) of measurements, performed in triplicate, from one experiment (from a set of n=3 separate experiments for cortisol and n=1 experiment for corticosterone and androstenedione). Statistically significant changes in steroid secretion with respect to basal secretion are noted by *** $P < 0.01$, calculated from triplicate incubations within each experiment.

Time-dependent CORTISOL Response					
	Response Ratio relative to basal				s.d.
	Expt. 1	Expt. 2	Expt. 3	Mean	
24 h VIP	1.7	4.1	1.6	2.47	1.42
48 h VIP	2.3	4.7	2.4	3.13	1.36
72 h VIP	3.0	7.5	2.9	4.47	2.63
96 h VIP	3.6	11.2	4.1	6.30	4.25

Time-dependent CORTICOSTERONE Response					
	Response Ratio relative to basal			Mean	s.d.
	Expt. 1				
24 h VIP	2.4			2.40	-
48 h VIP	1.9			1.90	-
72 h VIP	2.6			2.60	-
96 h VIP	3.2			3.20	-

Time-dependent ANDROSTENEDIONE Response					
	Response Ratio relative to basal			Mean	s.d.
	Expt. 1				
24 h VIP	1.1			1.10	-
48 h VIP	1.1			1.10	-
72 h VIP	1.1			1.10	-
96 h VIP	1.3			1.30	-

Table 4.2

Time-dependent changes in cortisol, corticosterone and androstenedione secretion in response to chronic treatment with VIP. Cells were treated with growth medium alone (basal) or containing VIP (10^{-7} M) for 24, 48, 72 or 96 h. Medium and agonists were replaced every 24 h. Medium from the last 24 h of the treatment period was collected and assayed for steroid content by separate RIAs. Steroid data was corrected for cellular protein. Results here are expressed as Response Ratios (response to stimulus / basal secretion) for individual experiments, triplicate incubations performed in each.

VIP also induced a significant rise in corticosterone secretion (relative to basal secretion) ($P < 0.01$, for triplicate incubations in 1 experiment), resulting in 2.4-fold, 1.9-fold, 2.6-fold and 3.2-fold increases in corticosterone secretion after 24, 48, 72 and 96 h treatment, respectively (see *Figure 4.1*).

An increase in androstenedione secretion above basal levels was not detected after 24, 48, 72 or 96 h VIP (10^{-7} M) treatment ($P \geq 0.05$, for triplicate incubations in 1 experiment) (see *Figure 4.1* and *Table 4.2*).

4.2.2 Cortisol response from H295R cells to 4 h treatment with VIP

Figure 4.2 shows the cortisol responses to short term (4 h) treatment with VIP. Cells were treated with AII (10^{-8} M) and forskolin (10^{-5} M) as positive controls and for comparison of responses.

A 4 h treatment with VIP (10^{-7} M) resulted in a $1.8 (\pm 0.4)$ -fold increase in cortisol secretion ($n=4$ individual experiments, $P < 0.05$ calculated from triplicate incubations within each experiment). This compared with a $2.1 (\pm 0.3)$ -fold increase following forskolin (10^{-5} M) treatment ($n=4$ individual experiments) and a $1.4 (\pm 0.4)$ -fold increase following AII (10^{-8} M) treatment ($n=4$ individual experiments).

4.2.3 Effect of pretreatment of H295R cells with AII or forskolin on the subsequent 4 h steroid responses to VIP

The cortisol, corticosterone and androstenedione responses to VIP (10^{-10} M to 10^{-7} M) following 96 h pretreatment of cells with AII or forskolin are shown in *Figure 4.3*. “Control” cells were treated in the same way as AII- or forskolin-pretreated cells, except that no agonist was added during the pretreatment period.

VIP (10^{-10} M to 10^{-7} M) evoked a concentration-dependent and significant increase in cortisol secretion from “control” cells ($P < 0.01$ for each concentration, calculated from triplicate incubations in 1 experiment). Concentrations of 10^{-8} M and 10^{-7} M VIP also caused a significant stimulation of corticosterone secretion ($P < 0.05$

and $P < 0.01$, respectively, calculated from triplicate incubations). Androstenedione levels were unaffected by any dose of VIP in “control” cells ($P \geq 0.05$, calculated from triplicate incubations) (see *Figure 4.3*).

VIP also stimulated cortisol and corticosterone secretion in a concentration-dependent manner from AII-pretreated H295R cells (see *Figure 4.3*). Chronic pretreatment of H295R cells with AII (10 nM) resulted in a marked increase in the corticosterone response to VIP. The threshold concentration for a significant ($P < 0.01$) stimulation of corticosterone secretion decreased to $\leq 10^{-9}$ M VIP, compared with $\leq 10^{-8}$ M in “control” cells (see *Figure 4.3*) and 10^{-8} M VIP elicited a 2.7-fold increase in corticosterone from AII-pretreated cells compared with a 1.7-fold increase in “control” cells. VIP still failed to stimulate androstenedione secretion over basal ($P \geq 0.05$, calculated from triplicate incubations in a single experiment) (see *Figure 4.3*).

Chronic forskolin (10 μ M) pretreatment greatly enhanced the subsequent cortisol and corticosterone concentration-responses to VIP in terms of both RR and total steroid output; for example, 10^{-8} M VIP caused secretion of 52 (± 3) pmol / mg protein / 4 h cortisol from “control” cells, which equated to a 2.3-fold increase over basal secretion, compared with cortisol secretion of 240 (± 42) pmol / mg protein / 4 h from forskolin-pretreated cells, with a RR of 6.9-fold. VIP (10^{-10} M to 10^{-7} M) caused a stimulation of androstenedione secretion following chronic forskolin pretreatment ($P < 0.01$ for all concentrations except 10^{-7} M, where $P < 0.05$, calculated from triplicate incubations in a single experiment) (see *Figure 4.3*). The threshold concentration of VIP required to stimulate androstenedione secretion was $\leq 10^{-10}$ M.

4.2.4 Effect of pretreatment of H295R cells with VIP on subsequent 4 h cortisol response to VIP

Having determined the effects of AII and forskolin pretreatment on subsequent VIP action, it was decided to investigate the effect of 96 h VIP pretreatment on the 4 h VIP response. The cortisol responses to 4 h VIP (10^{-9} M to 10^{-7} M) with or without VIP pretreatment are shown in *Figure 4.4* and *Table 4.3*.

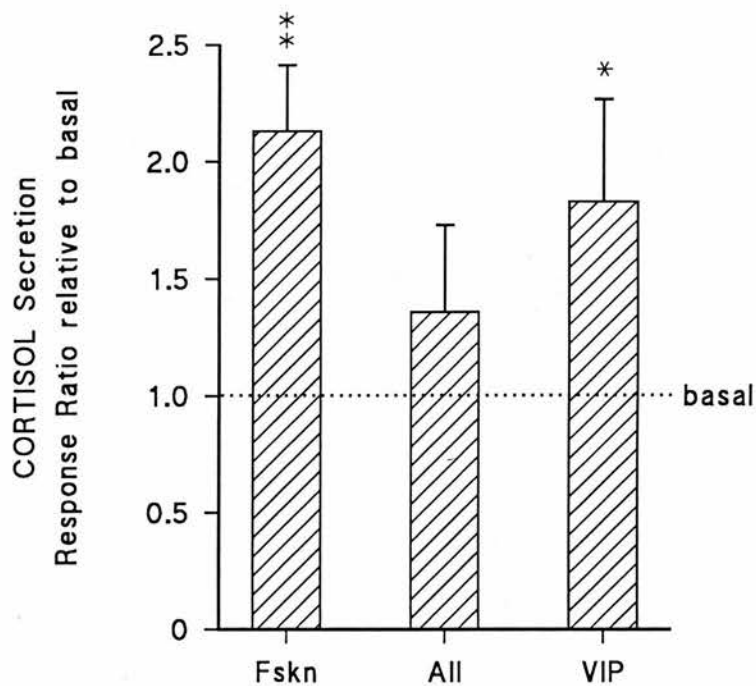
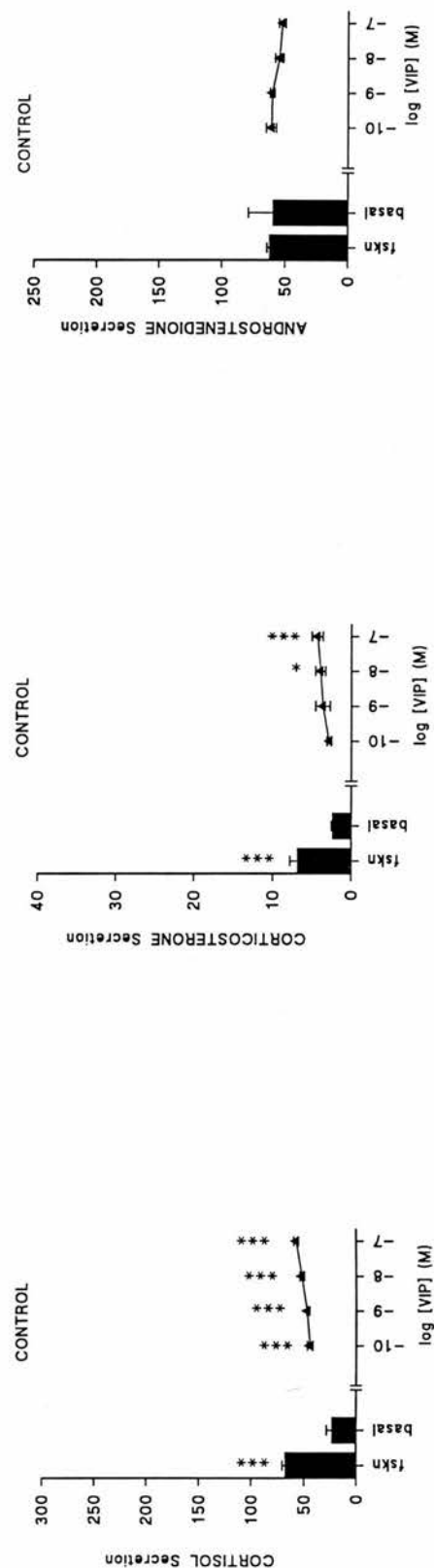


Figure 4.2

Cortisol secretion in response to short-term (4 h) treatment with VIP. Cells were treated with medium alone (basal, dotted line) or containing VIP (10^{-7} M), AII (10^{-8} M) or forskolin (10^{-5} M, fskn) for 4 h (cells were treated with forskolin and AII as a positive control and for comparison of responses). After 4 h, medium was removed and assayed for cortisol content by RIA. The results from n=4 separate experiments been combined and are presented as mean Response Ratios (response to stimulus / basal secretion) (\pm s.d.). Statistically significant changes in secretion compared with basal secretion within individual experiments are noted by ** $P < 0.02$ and * $P < 0.05$, in 4/4 experiments for fskn and VIP.

Figure 4.3

Effect of 96 h AII or forskolin preincubation of cells on the subsequent 4 h cortisol, corticosterone and androstenedione responses to VIP. Cells were pretreated with growth medium containing either AII (10 nM) or forskolin (10 μ M) for 96 h (medium and agonists were replaced every 24 h). “Control” cells were treated in exactly the same way but no agonist was added to the medium. After 96 h pretreatment, cells were washed thoroughly and incubated with either growth medium alone (solid bar labelled ‘basal’) or medium containing VIP (10⁻¹⁰, 10⁻⁹, 10⁻⁸ or 10⁻⁷ M, \blacktriangle) or forskolin (10⁻⁵ M, solid bar labelled ‘fskn’) for 4 h. After this time, medium was removed and assayed for cortisol, corticosterone and androstenedione by RIA. Steroid data was corrected for total cellular protein and expressed as “pmol / ml / mg protein / 4 h”. Results are presented as mean (\pm s.d.) from measurements, performed in triplicate, from a single experiment. Statistically significant changes in steroid secretion with respect to basal secretion are noted by *P<0.05 and ***P<0.01. Steroid responses from “Control” cells are shown below. For “AII pretreatment” and “Forskolin pretreatment” results, see next page.



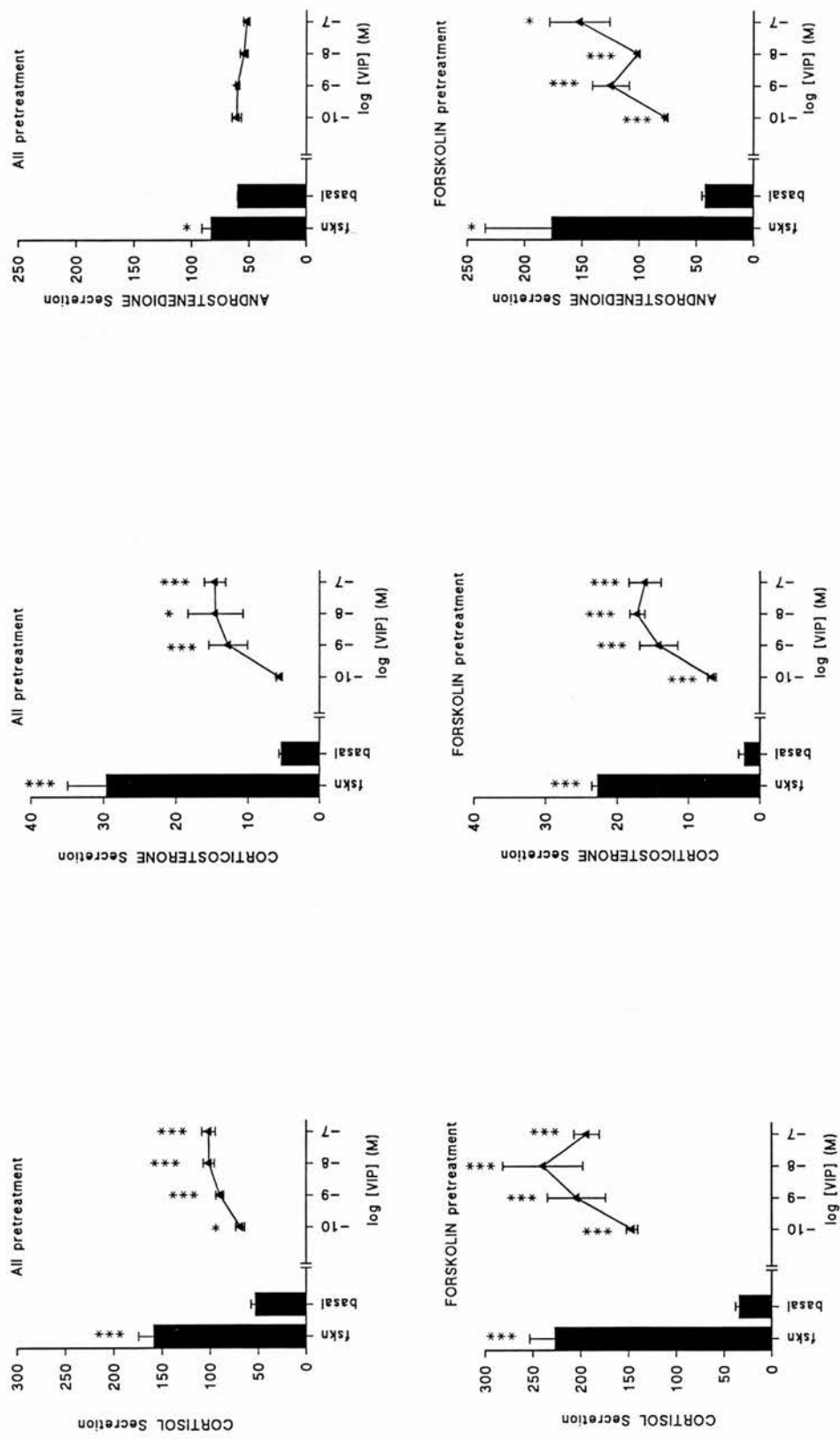


Figure 4.3 continued see previous page for figure legend.

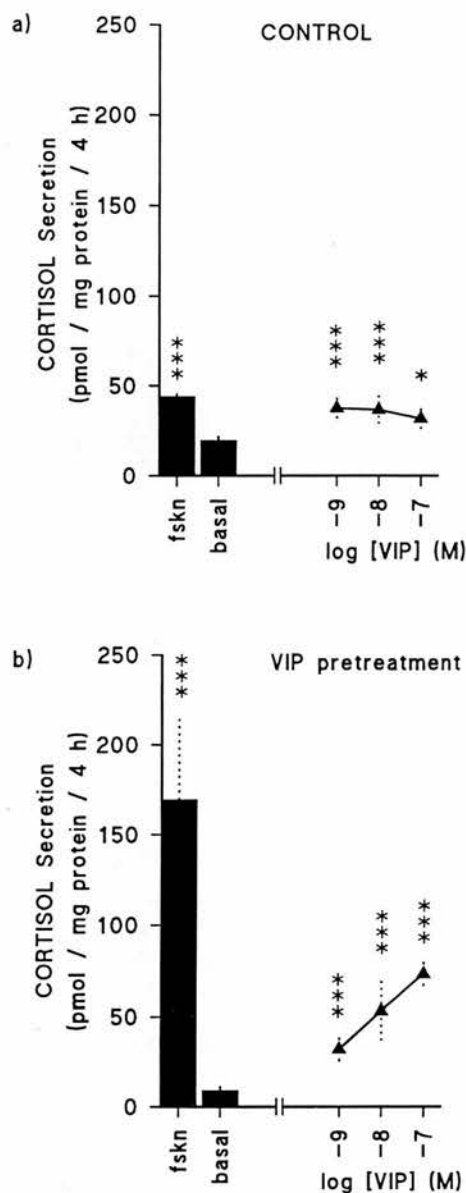


Figure 4.4

Effect of 96 h VIP preincubation of cells on the subsequent 4 h cortisol response to VIP. Cells were pretreated with growth medium alone ("control" cells) or containing VIP (10^{-7} M) for 96 h. Medium and agonists were replaced every 24 h. After 96 h pretreatment, cells were washed thoroughly and incubated with either growth medium alone (solid bar labelled 'basal') or containing VIP (10^{-9} , 10^{-8} or 10^{-7} M, \blacktriangle) or forskolin (10^{-5} M, bar labelled 'fskn') for 4 h. After this time, medium was removed and assayed for cortisol by RIA. Steroid data was corrected for total cellular protein. Combined data (mean (\pm range, i.e. min-max, ---)) from $n=2$ separate experiments, triplicate incubations performed in each, are shown. Statistically significant changes in steroid secretion, with respect to basal secretion, calculated from triplicate incubations within each experiment are noted by * $P<0.05$ and *** $P<0.01$. For data from each individual experiment see Table 4.3.

VIP (10^{-9} M to 10^{-7} M) significantly increased cortisol secretion from “control” cells ($P < 0.01$ for 10^{-9} M and 10^{-8} M VIP and $P < 0.05$ for 10^{-7} M VIP, calculated from triplicate incubations within each experiment, from $n=2$ separate experiments). However, the response was enhanced by prior 96 h treatment of the cells with VIP (10^{-7} M), a dose of 10^{-8} M VIP resulting in 5.3-fold and 6.3-fold stimulations of cortisol over basal ($n=2$ experiments, triplicate incubations performed in each) compared with a 1.6-fold and 2.0-fold increases, respectively, from “control” cells ($n=2$). The cortisol response to forskolin (10^{-5} M) was also greatly enhanced compared with “control” cells (30.5-fold and 10.9-fold compared with 2.4-fold and 2.1-fold for “VIP-pretreated” and “control” cells respectively ($n=2$, triplicate incubations performed in each)).

4.2.5 Optimising the pretreatment time with forskolin prior to 4 h VIP treatment

Figures 4.3 and 4.4 show that pretreating H295R cells with forskolin increased the subsequent cortisol response to VIP to a greater degree than preincubation with AII and to a similar degree as preincubation with VIP. It was decided to pretreat the cells with forskolin in all further VIP experiments. A time-course was performed to optimise the pretreatment time with forskolin (10 μ M). Although pretreatment of the cells was performed in serum-containing growth medium, serum-free growth medium was used for the subsequent 4 h VIP treatment in this experiment and all subsequent experiments with VIP.

Figure 4.5 shows the effect of 24, 48, 72 or 96 h forskolin pretreatment on the 4 h cortisol response to VIP (10^{-8} M). Total cortisol output was maximal after 72 h pretreatment in one experiment and 96 h in a second experiment, when 4 h treatment with VIP (10^{-8} M) resulted in a cortisol secretions of 52 (± 3.9) and 43 (± 5.4) pmol / mg protein / 4 h respectively ($n=2$ separate experiments, triplicate incubations performed in each) (see Figure 4.5). A time-dependent increase in cortisol responsiveness (in terms of n -fold increase (RR)) to VIP was observed following forskolin pretreatment (see Table 4.4). The responsiveness of the cells was similar

96 h CONTROL pretreatment				
	Mean (+/- s.d.) cortisol response to 4 h VIP (pmol / mg protein / 4 h)			
	<i>basal</i>	10^{-9} M VIP	10^{-8} M VIP	10^{-7} M VIP
<i>Expt. 1</i>	18 (1.4)	32 (3.4)	29 (3.1)	26 (4.4)
<i>Expt. 2</i>	22 (1.8)	43 (2.0)	44 (1.0)	37 (5.7)
mean	20	37.5	36.5	31.5
range	2	5.5	7.5	5.5

96 h VIP pretreatment				
	Mean (+/- s.d.) cortisol response to 4 h VIP (pmol / mg protein / 4 h)			
	<i>basal</i>	10^{-9} M VIP	10^{-8} M VIP	10^{-7} M VIP
<i>Expt. 1</i>	7 (0.5)	25 (1.6)	37 (4.9)	67 (12.3)
<i>Expt. 2</i>	11 (1.9)	38 (0.4)	69 (2.7)	80 (14.8)
mean	9	31.5	53	73.5
range	2	6.5	16	6.5

Table 4.3

Effect of 96 h VIP pretreatment of H295R cells on cortisol response to subsequent 4 h treatment with VIP, compared with 'control' cells. Cells were pretreated with growth medium alone ('control') or containing VIP (10^{-7} M) for 96 h. Medium and agonists were replaced every 24 h. Following the pretreatment period, cells were washed thoroughly and treated with serum-free medium alone (basal) or containing VIP (10^{-9} M, 10^{-8} M, 10^{-7} M) for 4 h. Medium was then collected and assayed for cortisol content by RIA. Steroid data was corrected for cellular protein. Results here are expressed as mean (\pm s.d.) cortisol secretion (pmol / mg protein / 4 h), calculated from triplicate incubations within each experiment of n=2 separate experiments.

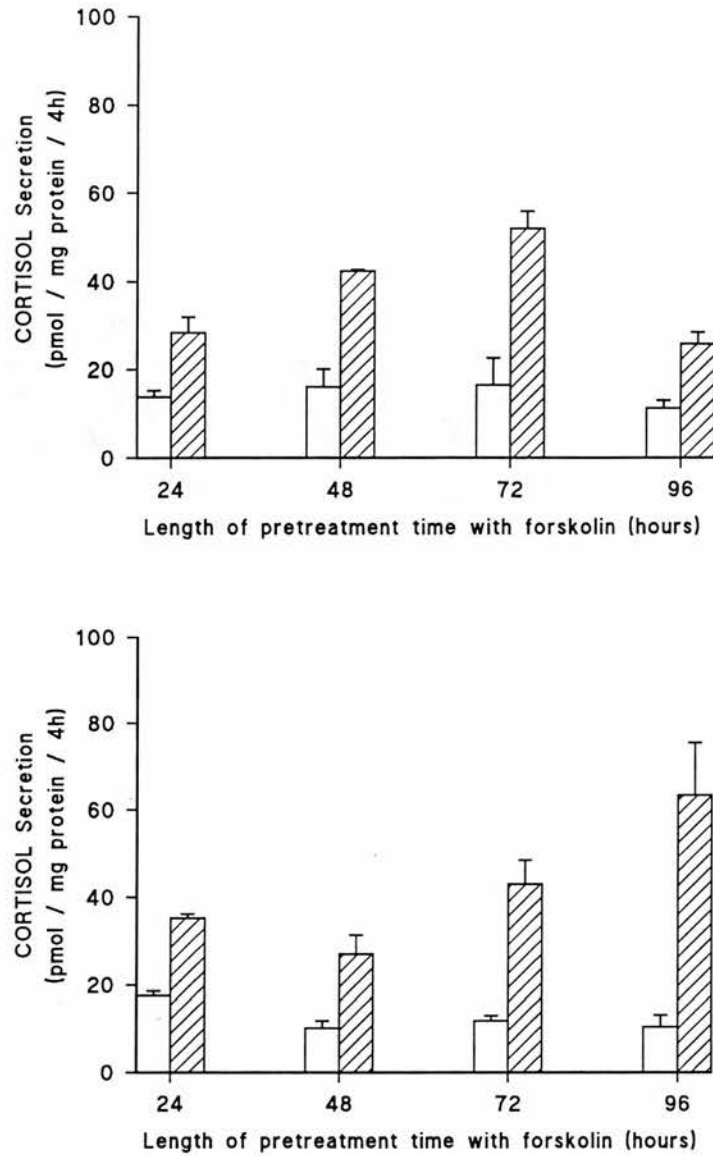


Figure 4.5

Time-courses to determine the optimum length of time to pretreat cells with forskolin in order to enhance the cortisol response to subsequent 4 h treatment with VIP. Cells were pretreated with forskolin (10 μ M) for 24, 48, 72 or 96 h. After the defined pretreatment period, cells were washed thoroughly and then incubated with serum-free growth medium alone (basal, open bars) or containing VIP (10⁻⁸ M, hatched bars) for 4 h. After this time, medium was collected and assayed for cortisol content by RIA. Data was corrected for cellular protein. Results presented are mean (\pm s.d) of data, calculated from triplicate incubations, from 2 separate experiments.

CORTISOL response to VIP (RR)				
	Forskolin pretreatment time (hours)			
	24	48	72	96
<i>Expt.1</i>	2.07	2.64	3.15	2.30
<i>Expt.2</i>	2.00	2.66	3.62	6.05
Mean	2.04	2.65	3.39	4.18

Table 4.4

Effect of increasing length of pretreatment time with forskolin on the subsequent cortisol response to VIP. Cells were pretreated with growth medium alone ("control") or containing forskolin (10 μ M) for 24, 48, 72 or 96 h. After pretreatment, cells were washed thoroughly and then treated with serum-free growth medium containing VIP (10^{-8} M) for 4 h. After this time, medium was collected and assayed for cortisol content by RIA. Steroid data was corrected for total cellular protein. Results in this table are expressed as Response Ratios (response to stimulus / basal secretion, RR) from n=2 separate experiments, triplicate incubations performed in each.

after 72 h and 96 h pretreatment, when 10^{-8} M VIP elicited 3.4 (range ± 0.2)-fold and 4.2 (range ± 1.9)-fold increases in cortisol secretion over basal, respectively (n=2, triplicate incubation performed in each). A pretreatment time of 72 h was chosen for subsequent experiments.

The increase in cell responsiveness to VIP following 72 h forskolin pretreatment was confirmed (see *Table 4.5*). A dose of 10^{-8} M VIP resulted in a 3.6 (± 0.6)-fold stimulation of cortisol secretion over basal from forskolin-pretreated cells (n=4). This compared with a 2.0 (± 0.3)-fold increase over basal from “control” cells (n=4).

72 h CONTROL pretreatment			
	Cortisol Response Ratio to 4 h VIP		
	10^{-8} M VIP	3.3×10^{-8} M VIP	10^{-7} M VIP
Expt.1	2.18	2.17	1.67
Expt.2	2.03	2.54	1.70
Expt.3	1.64	2.05	1.48
Expt.4	2.25	2.62	2.47
Mean	2.03	2.35	1.83
s.d.	0.27	0.28	0.44

72 h FORSKOLIN pretreatment			
	Cortisol Response Ratio to 4 h VIP		
	10^{-8} M VIP	3.3×10^{-8} M VIP	10^{-7} M VIP
Expt.1	2.97	2.85	4.01
Expt.2	3.57	4.09	3.42
Expt.3	4.31	4.94	4.38
Expt.4	3.44	3.75	3.71
Mean	3.57	3.91	3.88
s.d.	0.56	0.86	0.41

Table 4.5

Effect of 72 h pretreatment of H295R cells with forskolin on the subsequent 4 h cortisol response to VIP. Cells were pretreated with forskolin (10 μ M) for 72 h, the medium and agonist replaced every 24 h. "Control" cells were treated in exactly the same way except that no forskolin was added to the incubation medium. After pretreatment, the cells were washed thoroughly and then treated with serum-free growth medium alone (basal) or containing VIP (10^{-8} M, 3.3×10^{-8} M or 10^{-7} M) for 4 h. After this time, medium was collected and assayed for cortisol content by RIA. Steroid data was corrected for total cellular protein. The results are expressed as Response Ratios (response to stimulus / basal response) calculated from triplicate incubations from n=4 separate experiments.

4.3 Discussion

VIP directly stimulated steroid production from H295R cells in a dose-dependent and time-dependent manner. The steroid responses of the cells to AII (10^{-8} M) and forskolin (10^{-5} M) were similar to those presented in chapter 3.0, except that the 24 h AII-induced corticosterone response did not reach significance ($P \geq 0.05$) in this set of experiments.

Similar to AII and forskolin, 24 h VIP treatment caused an increase in cortisol and corticosterone secretion but not androstenedione production (see *Table 4.1*). The cortisol response to VIP was of a lesser magnitude than that elicited by either AII or forskolin. This is unlikely to be due to a desensitisation of the steroid responses to VIP because the cortisol response continued to increase up to 96 h VIP exposure (see *Figure 4.1*). Thus, VIP exerts a clear steroidogenic effect in H295R cells, though is a less potent chronic (24 h) agonist than AII or forskolin.

Chronic (24 - 96 h) VIP treatment progressively enhanced the cortisol responsiveness of the cells (in that the RR increased with time), similar to that seen in response to forskolin (Chapter 3.0 and *Figure 4.1*), though VIP was a less potent chronic agonist than forskolin over the 96 h treatment period (*Table 4.2* and *Table 3.1*). This observation raises the possibility that chronic VIP treatment increases the expression of key steroidogenic enzymes. Indeed, VIP has been shown to upregulate expression of P450scc and P450c17 mRNAs in hen granulosa cells (Johnson *et al*, 1994) and P450scc synthesis in cultured rat ovarian granulosa cells (Trzeciak *et al*, 1986).

Unlike cortisol, the chronic corticosterone responsiveness of H295R cells to VIP did not increase between 24 - 96 h treatment (see *Figure 4.1*). This differed from the chronic corticosterone response to AII or forskolin, where the sensitivity of the cells increased with time following AII or forskolin treatment (as described in Chapter 3.0).

These steroidogenic effects of VIP meant that the ratios of cortisol / corticosterone and cortisol / androstenedione increased with time. Corticosterone /

androstenedione remained relatively constant with increasing time of VIP treatment. Since VIP can alter the expression of P450scc and P450c17 in ovarian tissue (Johnson *et al*, 1994; Trzeciak *et al*, 1986), it is possible that chronic VIP treatment can modulate the steroid phenotype of this cell-line in a similar way to AII or forskolin (see chapter 3.0).

Short-term (4 h) treatment with VIP led to a concentration-dependent increase in cortisol and corticosterone secretion (see *Figure 4.3*). As with chronic VIP treatment, androstenedione secretion was unaffected by short-term VIP treatment.

The 4 h corticosterone response to VIP was greatly enhanced following prior 96 h treatment with AII, as evidenced by the decrease in threshold concentration of VIP from 10^{-8} M in “control” cells to 10^{-9} M in “AII-pretreated” cells (see *Figure 4.3*). The responsiveness of the cells for cortisol secretion actually decreased slightly after AII-pretreatment. This pattern of response would be consistent with a shift in steroidogenic phenotype to a zg-like cell by chronic AII pretreatment (as discussed in Chapter 3.0). Androstenedione secretion remained unaffected by VIP, in keeping with this argument.

Following forskolin pretreatment, 4 h VIP caused a concentration-dependent increase in the secretions of all 3 steroids (see *Figure 4.3*). VIP was found to stimulate androstenedione secretion from H295R cells only after chronic forskolin pretreatment, consistent with the activation of the androgen pathway being highly dependent on cAMP in this cell-line. This contrasts with the lack of effect of VIP on androstenedione following pretreatment of the cells with AII (a “non-cAMP” agonist). VIP-mediated androstenedione secretion has been demonstrated from isolated perfused porcine adrenals (Bornstein *et al*, 1993) and from cultured human adrenal cells (Bornstein *et al*, 1996), though indirect mechanisms of action were attributed to VIP. This study suggests that, if VIP binding-sites are present on zfr cells, VIP may also regulate androstenedione production directly.

The sensitivity of the cells to VIP markedly increased following forskolin pretreatment, with an increase in Response Ratios for all 3 steroids and a decrease in the threshold concentration for corticosterone secretion, from 10^{-8} M in “control”

cells to 10^{-10} M (or less) in “forskolin-pretreated” cells (see *Figure 4.3*). Possible explanations include an upregulation of VIP receptors at the cell-surface or alternatively an upregulation of steroidogenic enzyme expression. Evidence for the latter explanation is that forskolin pretreatment also enhanced the steroid responses to a subsequent 4 h re-challenge with forskolin. Indeed, forskolin has been shown to markedly increase the expression of a number of key steroidogenic enzymes in this cell-line (as discussed in Chapter 3.0). Although an accompanying increase in cell-surface VIP receptors cannot be excluded, it seems possible that the increased sensitivity of the cells to VIP reflects an overall increase in responsiveness of the cells to steroidogenic agonists resulting from increased enzyme expression induced by forskolin pretreatment.

A 96 h pretreatment of H295R cells with VIP itself also increased the subsequent cortisol response to acute VIP treatment, compared with “control” cells (see *Figure 4.4*). The 4 h response to forskolin was also enhanced by chronic VIP pretreatment, consistent with VIP modulating steroidogenic enzyme expression.

On the basis of these results, H295R cells were pretreated with forskolin prior to short-term VIP treatment in subsequent experiments. Studies were first undertaken to determine the optimum length of time to pretreat the cells with forskolin (see *Figure 4.5*). A pretreatment time of 72 h was chosen on the basis of steroid output (pmol / mg protein / 4 h), steroidogenic capacity (RR) and overall convenience of experimental time-length (see *Figure 4.5* and *Table 4.4*). The increased responsiveness (RR to 4 h VIP treatment) of the cells following 72 h forskolin pretreatment, compared with “control” cells, was then confirmed in a series of experiments (see *Table 4.5*).

In summary, extensive evidence exists that VIP modulates steroid secretion from the adrenal. Whether VIP exerts this effect (in part) directly on adrenocortical cells has been unclear until now. In this study, VIP directly stimulated cortisol, corticosterone and androstenedione production from H295R cells. Moreover, VIP appeared to be a potent stimulator of steroidogenesis in these cells. Bornstein *et al* (1996) obtained comparable results with human adrenal cells in culture which

secreted cortisol and androstenedione, as well as DHEA, testosterone and aldosterone, in response to VIP. Although these authors attributed the response to an indirect effect involving catecholamine release, the studies presented in this chapter clearly show that VIP has a direct action on steroidogenesis by adrenocortical cells, an effect that may have been masked in primary cultures of adrenocortical cells, which are likely to contain contaminating chromaffin cells.

Interestingly, a direct, receptor-mediated steroidogenic action for VIP on the *zg* of rat adrenals has recently been reported (Hinson *et al.* 1996; Hinson & Kapas, 1997). These receptors may be regulated by sodium status *in vivo* because a low sodium diet in rats resulted in increased aldosterone response to VIP, compared with a control sodium diet (Hinson & Kapas, 1995), and also increased VIP receptor binding and augmented adrenal content of VIP (Hinson *et al.*, 1996). The possible physiological importance of a direct action of VIP on adrenocortical steroidogenesis is further discussed in chapter 8.0.

As developed further in this thesis, the H295R cell-line is of use in further characterising this direct VIP action, for example, in terms of second messenger systems activated and receptor subtypes involved.

5.0 Characterisation of the cortisol and second messenger responses from H295R cells to VIP

5.1 Introduction

A number of intracellular signalling mechanisms and second messengers have been implicated in the regulation of adrenal steroidogenesis, including: 1) the adenylate cyclase-cAMP system, 2) phospholipase C (PLC)-mediated inositol trisphosphate and diacylglycerol (DAG) formation, 3) the guanylate cyclase-cGMP system, 4) phospholipase A₂-induced release of arachidonic acid and formation of eicosanoids, 5) phospholipase D-induced cleavage of phospholipids generating phosphatidic acid and DAG, 6) calcium fluxes.

In this chapter, three of these second messengers were investigated following treatment of H295R cells with VIP. These were: 1) cAMP production, 2) cGMP production and 3) phosphoinositide turnover. These second messengers were selected on the basis that they have been shown to be stimulated by VIP in other tissues (section 5.1.4). The formation of each of these three second messengers will be reviewed in the following sections.

5.1.1 Adenylate cyclase-cAMP system

The earliest signalling mechanism identified in adrenocortical cells was the adenylate cyclase-cAMP system (Sutherland & Rall, 1958). Adenylate cyclase (AC) is a hormone-sensitive enzyme which catalyses the conversion of adenosine-5'-trisphosphate (ATP) to the intracellular second messenger molecule, cyclic-3',5'-monophosphate (cAMP) (*Figure 5.1*). To date, at least nine isoforms of AC have been identified; the regulatory properties of each may vary. In particular, it now appears that Ca²⁺ / protein kinase C (PKC) is important in the regulation of several of these isoforms (reviewed by Antoni, 1997), leading to cross-talk between receptors which activate these different systems, for example, ACTH receptors (which are predominantly associated with increased cAMP production) and AII receptors (which

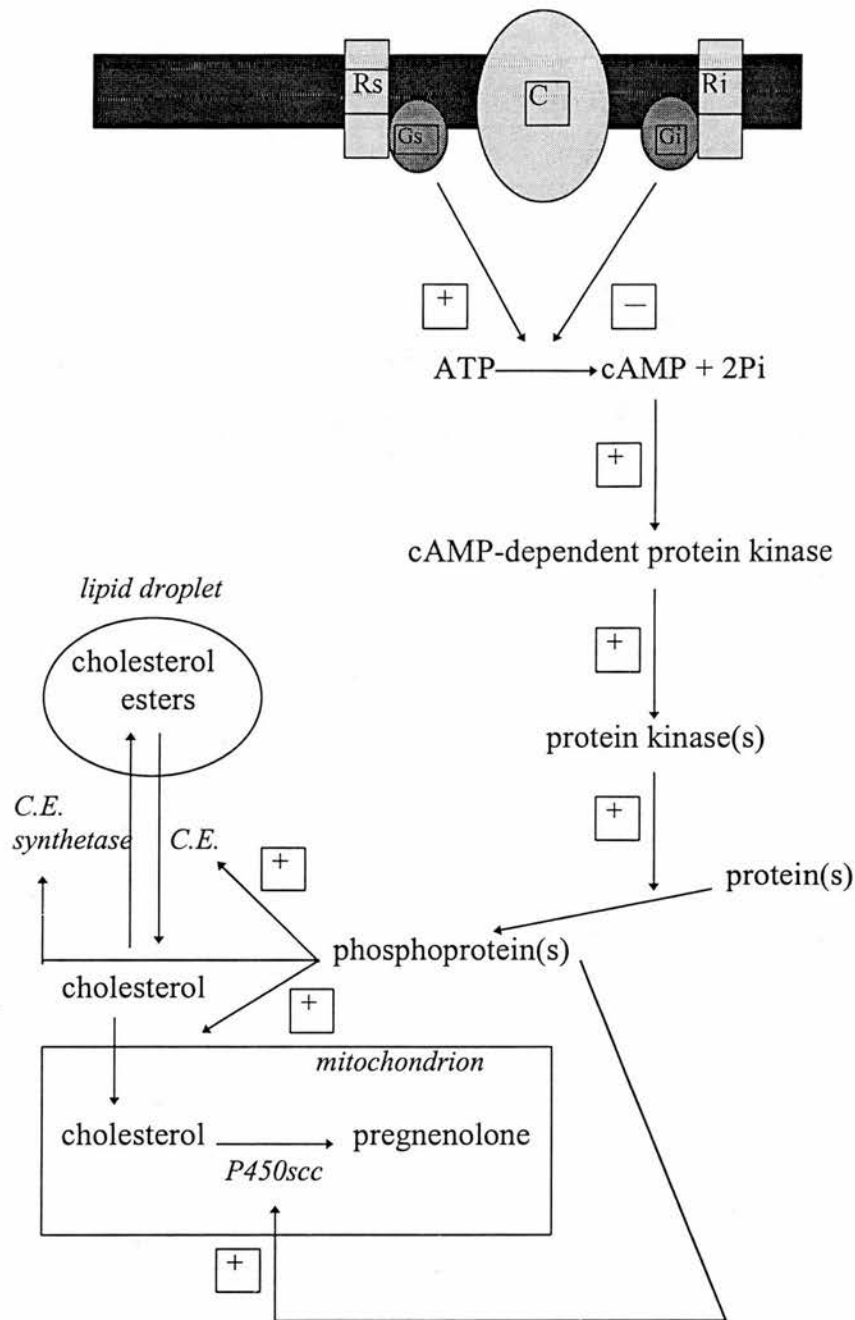


Figure 5.1

Structure of the hormonally regulated adenylate cyclase. Agonists, for example, ACTH, interact with stimulatory receptors (R_S) which activate the enzyme catalytic unit (C) via a pertussis-toxin insensitive G protein (G_S). The enzyme can also be inhibited by agonists which interact with receptors linked to inhibitory G proteins (G_I). cAMP, formed from ATP, stimulates cAMP-dependent protein kinases, eventually leading to activation of steroidogenesis through the phosphorylation of specific enzymes (P450_{scc}).

have been linked to increased intracellular Ca^{2+} and PKC activation). So far, the AC3 isoform has been detected in bovine zg cells (Burnay *et al*, 1998).

cAMP further transmits the intracellular signal by activating the cAMP-dependent protein kinase, protein kinase A, leading to subsequent phosphorylation events. PKA is a tetrameric enzyme, which exists in multiple isoforms, and consists of two catalytic subunits (C), which specifically phosphorylate serine and threonine residues, and two regulatory subunits (R) (Doskeland *et al*, 1993). The importance of cAMP-dependent protein kinase in adrenal steroidogenesis was demonstrated by Wong *et al* (1986) using mutant cells which lacked cAMP-dependent protein kinase activity. Transformation of the cells with DNA from an ACTH- and cAMP-responsive adrenocortical cell-line resulted in the recovery of a steroid response to ACTH and 8-bromo-cAMP (Wong *et al*, 1986).

Changes in AC activity occur after hormone-binding and activation of an AC-coupled receptor, for example, the ACTH receptor. This results in the activation of a guanine nucleotide-binding regulatory protein (G-protein), which couples the hormone-sensitive receptor to AC. The structure and function of G-proteins in the activation of AC was reviewed by Gilman (1995). In summary, each G-protein consists of three subunits, designated α , β and γ . The β and γ subunits are highly homologous between each specific G-protein, whereas the α -subunits show more structural diversity. Indeed, the nomenclature for each specific G-protein is designated by the α -subunit of the molecule. With respect to AC, members of two subfamilies of G-protein are directly regulatory: 1) the G_s group, members of which stimulate AC activity and 2) the G_i group, members of which inhibit AC activity.

Activation of the G-protein involves displacement of GDP by GTP from the α -subunit of the G-protein molecule, followed by dissociation of the α -subunit from the β and γ subunits, which remain associated as a $\beta\gamma$ complex. The α and $\beta\gamma$ subunits are capable of altering AC activity by binding to the enzyme. The α -subunits of the G_s family generally stimulate AC activity whereas the α -subunits of certain members of the G_i family inhibit AC activity. The effect of the $\beta\gamma$ complex depends on the type of AC involved; for example, $\beta\gamma$ enhances activity of AC2,

whilst inhibiting the activity of AC1 (Tang & Gilman, 1991). The process ends when the intrinsic GTPase activity of the α -subunit hydrolyses the bound GTP to GDP. The GDP- α -subunit re-associates with the $\beta\gamma$ -subunits and basal levels of AC activity are then restored.

Whilst the G_s and G_i subfamilies of G-proteins exert a direct action on adenylate cyclase activity, other G-proteins can influence cAMP production. For example, AII receptor-mediated stimulation of G_q in adrenal zg cells (Smrcka *et al*, 1991) ultimately results in PKC activation, which modulates adrenal cell AC activity such that a further increase in cAMP production occurs (Baukal *et al*, 1994). Thus, AII and ACTH can synergistically increase cAMP levels in adrenocortical zg cells.

Intracellular cAMP is degraded by phosphodiesterases; the activities of these enzymes in different tissues are important in the modulation of cAMP signalling (Conti *et al*, 1995).

5.1.2 Guanylate cyclase-cGMP system

Guanylate cyclase (GC) is a ubiquitous enzyme which converts guanosine-5'-triphosphate (GTP) to cyclic guanosine-3',5'-monophosphate (cGMP), an intracellular second messenger. GC has been shown to exist in a cytosolic (soluble) form and a membrane-associated (particulate) form.

The structure and mechanism of activation of soluble guanylate cyclase (sGC) is reviewed by Hobbs (1997). The enzyme exists as haem-containing heterodimers containing an α and β subunit. Several isoforms of sGC have been cloned, comprising different combinations of α -subunit isoforms and β -subunit isoforms (so far discovered; α_1 , α_2 , (α_3) and β_1 , β_2 , (β_3)). Although both α and β subunits contain catalytic sites at their C-termini, dimerisation of the α and β subunits is obligatory for catalytic activity of the enzyme (Hobbs, 1997, for review).

sGC is most potently activated by nitric oxide (NO), formed from NO synthase (NOS) action on L-arginine (Mayer & Andrew, 1998, for review). Three

isoforms of NOS have currently been identified (nNOS; found in brain and central nervous system, eNOS: found in endothelial cells and iNOS; inducible form of NOS).

The N-terminal haem-moiety of sGC is responsible for conferring NO-sensitivity to the enzyme. Oxidation of the haem group results in loss of enzyme activity whilst binding of NO to the haem group of sGC results in formation of a pentacoordinate nitrosyl-haem complex and activation of the enzyme (Hobbs, 1997, for review).

The particulate form of GC (pGC) is a transmembrane protein, associated with organelle membranes as well as cell-surface membranes. As with sGC, several isoforms are thought to exist (GC-A-F) (Wedel & Garbers, 1997). An important activator of pGC, with respect to the adrenal, is ANP (Anand-Srivastava & Trachte, 1993, for review). ANP binds to the NH₂-terminal, extracellular, peptide binding domain of the enzyme, resulting in activation of the intracellular catalytic domain without the involvement of G-proteins. The intracellular domain contains a protein kinase-like region, as well as a catalytic region, which is necessary for ANP control of pGC activity (Anand-Srivastava & Trachte, 1993).

cGMP further transmits the intracellular signal by actions on cGMP-dependent protein kinases (Lohmann *et al*, 1997), cGMP-dependent phosphodiesterases (Beavo, 1995) and cyclic nucleotide-gated ion channels (Biel *et al*, 1998).

5.1.3 Phosphoinositide (PI) metabolism

Phosphoinositides constitute approximately 2 - 8% of cell membrane phospholipids. Hormone-stimulated turnover of phosphoinositides was first described by Hokin & Hokin (1953), following the observation of increased ³²P incorporation into phospholipids of pancreatic exocrine cells in response to cholinergic stimulation. Michell (1975) later suggested that this agonist-mediated rise in phospholipid turnover was linked to increases in intracellular Ca²⁺, leading to

the ultimate recognition of inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃) (released from phospholipase C (PLC) action on phosphoinositide 4,5-bisphosphate) as an important intracellular second messenger and regulator of cytosolic Ca²⁺ concentrations (Streb *et al*, 1983; Berridge & Irvine, 1984). A second effect of PLC action on phosphoinositide substrate is release of the product DAG, an activator of PKC.

The major pathways of phosphoinositide metabolism are shown in *Figure 5.2*. The two important metabolites of this pathway (which serve as second messengers), Ins(1,4,5)P₃ and 1,2-diacylglycerol (DAG), are formed by PLC-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂). PLC activity is increased following agonist binding to specific receptors, for example, the AII receptor in adrenocortical zg cells. Evidence suggests that this effect is mediated via a G-protein, G_q (Smrcka *et al*, 1991). Whilst Ins(1,4,5)P₃ is responsible for Ca²⁺ mobilisation, DAG activates the Ca²⁺-dependent protein kinase C (PKC) (Berridge & Irvine, 1989, review).

Rapid inactivation of Ins(1,4,5)P₃ to free inositol occurs via 1) sequential dephosphorylation by phosphatases, forming Ins(1,4)P₂, then Ins(4)P, then free inositol or 2) conversion to Ins(1,3,4,5)P₄ by Ca²⁺/calmodulin-dependent cytosolic 3-phosphokinase. The latter pathway may be predominant as 3-phosphokinase has a greater affinity for Ins(1,4,5)P₃ than the 5-phosphatase which degrades it to Ins(1,4)P₂.

Ins(1,3,4,5)P₄ may also promote Ca²⁺ influx / mobilisation (Ely *et al*, 1990) prior to conversion to the relatively inactive Ins(1,3,4)P₃, subsequent dephosphorylation to inositol monophosphates and final degradation by a non-specific, lithium-sensitive, monophosphatase to yield free inositol. Inclusion of lithium in experimental media allows the accumulation of inositol phosphates for assay. Other tetrakisphosphates (Ins(1,3,4,6)P₄ and Ins(3,4,5,6)P₄) as well as InsP₅ and InsP₆ may be formed in adrenocortical cells (Balla *et al*, 1989a).

DAG is phosphorylated by DAG-kinase to form phosphatidic acid (PtdOH), which is then converted to phosphatidylinositol (PtdIns) via cytidine-

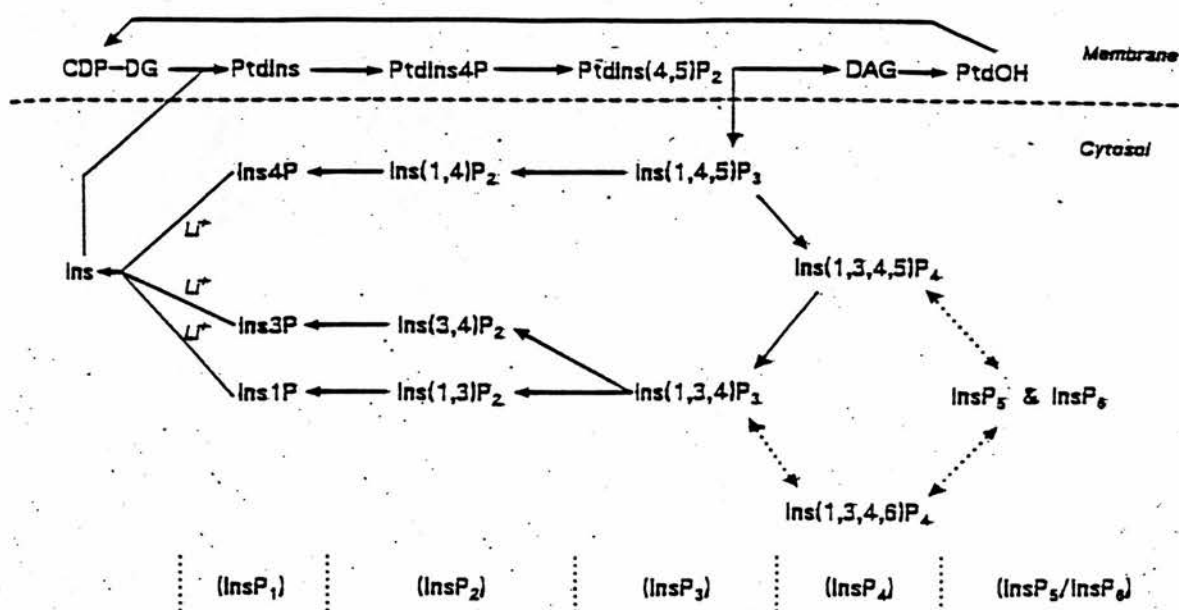


Figure 5.2

An outline of the major metabolic pathways which are activated as a consequence of phospholipase C action on PtdIns(4, 5)P₂ to produce the second messengers, Ins(1, 4, 5)P₃ and DAG. The final steps in the recycling of Ins(1, 4, 5)P₃ to free inositol (Ins) can be inhibited by lithium, leading to accumulation of phosphoinositols.

diphosphodiacylglycerol (CDP-DAG) combination with free inositol. The phosphoinositides PtdIns(4)P and PtdIns(4,5)P₂ are formed from PtdIns by sequential phosphorylation of the inositol ring by specific kinases.

5.1.3.1 *Ins(1,4,5)P₃ and DAG in adrenocortical cells*

The hydrolysis of phosphoinositides in bovine and rat zg cells (Kojima *et al*, 1985; Farese *et al*, 1984) as well as bovine zfr cells (Bird *et al*, 1989) in response to AII has been demonstrated and this effect has been linked to steroid secretion (see section 3.1.2 for mechanism of AII action in adrenocortical cells). However, in ovine zfr cells, AII failed to stimulate steroidogenesis whilst still causing an increase in phosphoinositide turnover (Viard *et al*, 1990). Ins(1,4,5)P₃ was produced in a biphasic manner in cultured bovine zg cells (Balla *et al*, 1989b), and the secondary increase was associated with an increase in Ins(1,3,4,5)P₄. Distinct receptors for Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ have also been detected on bovine adrenal microsomes (Guillemette *et al*, 1987; Enyedi & Williams, 1988). An AII-induced biphasic increase in DAG formation has also been described (Hunyady *et al*, 1990).

DAG activates PKC, an enzyme first identified by Nishizuka (1984) which also requires phospholipid and Ca²⁺ for its activation. PKC activity has been detected in bovine and rat adrenocortical cell preparations (Coyne *et al*, 1986; Widmaier & Hall, 1985). In addition, PKC has been shown to phosphorylate the first enzyme of steroidogenesis, P450scc (Vilgrain *et al*, 1984).

Agonist-stimulated phosphoinositide turnover and regulation of steroidogenesis has been reviewed by Bird *et al* (1990b). Kojima *et al* (1985) demonstrated that sustained AII-induced steroidogenesis from superfused bovine zg cells could only be fully reproduced by co-administration of the Ca²⁺ ionophore, A23187, and the phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA; an activator of PKC), suggesting that both Ca²⁺ influx and PKC activation are required for prolonged stimulation of steroidogenesis by PLC agonists.

5.1.4 Second messenger systems activated by VIP

The best characterised and most commonly observed intracellular response following VIP administration is activation of AC and increased cAMP production. Couvineau *et al* (1990) demonstrated VIP receptor coupling to G_s in rat liver and VIP has been shown to increase cAMP levels in cerebral cortex (Magistretti & Schorderet, 1984), thyroid (Siperstein *et al*, 1988), liver, pituitary (Rostene, 1984), gastric smooth muscle cells (Jin *et al*, 1993), mouse N1E-115 neuroblastoma cells, PC12 cells (Wessels-Reiker *et al*, 1991) and in a human colon carcinoma cell-line (LoVo) (Yu *et al*, 1992) amongst others.

Whilst in many tissues VIP appeared to stimulate cAMP production alone, in some cell types (for example, guinea pig / rabbit gastric smooth muscle cells and rat pinealocytes), it also caused an increase in cGMP levels (Jin *et al*, 1993; Murthy *et al*, 1993, 1995; Schaad *et al*, 1995). Studies by Murthy *et al* (1993) on dispersed rabbit gastric muscle cells indicated that this action involved VIP-specific G-protein-coupled receptors, distinct from those which activate AC, which stimulated Ca^{2+} influx (through a methoxyverapamil-sensitive Ca^{2+} channel), leading to activation of a constitutive NOS, NO generation, GC activation, cGMP synthesis and cGMP-dependent protein kinase activation. In rat pinealocytes, VIP also stimulated cGMP production via NO and activation of sGC (Spessert, 1993), followed by an increase in intracellular Ca^{2+} , apparently via a cGMP-gated, *l-cis*-diltiazem-sensitive, rod-type cation channel (Schaad *et al*, 1995).

Increased phosphoinositide turnover has been demonstrated following VIP treatment of rat superior cervical ganglia (Audigier *et al*, 1986). In addition, VIP increased $Ins(1,4,5)P_3$ in the adrenal medulla (Malhotra, 1988) and activated PKC in nuclei isolated from rat hippocampus (Weill, 1989).

VIP has been shown to depolarise retinal horizontal neurons via a cAMP-independent mechanism (Lasater *et al*, 1983). In cultured rat zf cells, VIP modulated T-type Ca^{2+} channels via a PKA-independent mechanism (Barbara & Takeda, 1995). VIP also modulated membrane potentials of spinal cord dorsal root terminals and

motoneurons (Phillis *et al*, 1978) and submucosal plexus neurons (Mihara *et al*, 1985).

In this chapter, the cortisol response to VIP from H295R cells was further characterised and accompanying activation of the AC-cAMP, GC-cGMP and PI second messenger systems was investigated. Cells were pretreated with forskolin (10 μ M) for 72 h, to enhance the subsequent cortisol response, as discussed in chapter 4.0. Following the pretreatment period, all subsequent treatments with VIP were performed in serum-free medium.

5.2 Results

5.2.1 Concentration-dependent cortisol secretion from the H295R cell-line in response to VIP

In order to determine the characteristics of the VIP-induced cortisol response from the H295R cell-line, the cells were treated with a range of concentrations of VIP from 10^{-11} M to 10^{-7} M VIP for 4 h. *Figure 5.3* shows the resulting concentration-dependent cortisol secretion from the H295R cells, reaching a maximum 3.9 (± 0.9)-fold increase by 3.3×10^{-8} M ($n=4$ individual experiments), the threshold effect occurring at 10^{-11} M ($n=4$ individual experiments). The pEC50 value (negative logarithm to the base 10 of the concentration (Molar) producing a half-maximal response) obtained was 9.22 (± 0.43) ($n=4$ individual experiments). The maximal cortisol effect of VIP was equivalent to that produced by 10 μ M forskolin.

5.2.2 Second messenger pathways activated by VIP in H295R cells

5.2.2.1 cAMP production following treatment of H295R cells with VIP

(a) Time-course of cAMP production in response to VIP

Since a concentration of 3.3×10^{-8} M VIP induced a maximal cortisol response in H295R cells, this concentration was used to determine a time-course for VIP-induced cAMP accumulation in the overlying medium (see *Figures 5.4a and 5.4b*). Levels of cAMP in the medium remained steady in control cells (treated with serum-free growth medium alone, indicated as 'basal' in *Figures 5.4a and 5.4b*), whereas VIP treatment resulted in a significant and time-dependent increase in the amount of cAMP in the overlying medium. Accumulation of cAMP in the medium increased slowly up to 10 minutes exposure to VIP (*Figure 5.4a*), thereafter rising more steeply with longer VIP treatment (*Figure 5.4b*). Levels of cAMP measured were still increasing after 4 h VIP treatment. Because cells were stimulated for 4 h

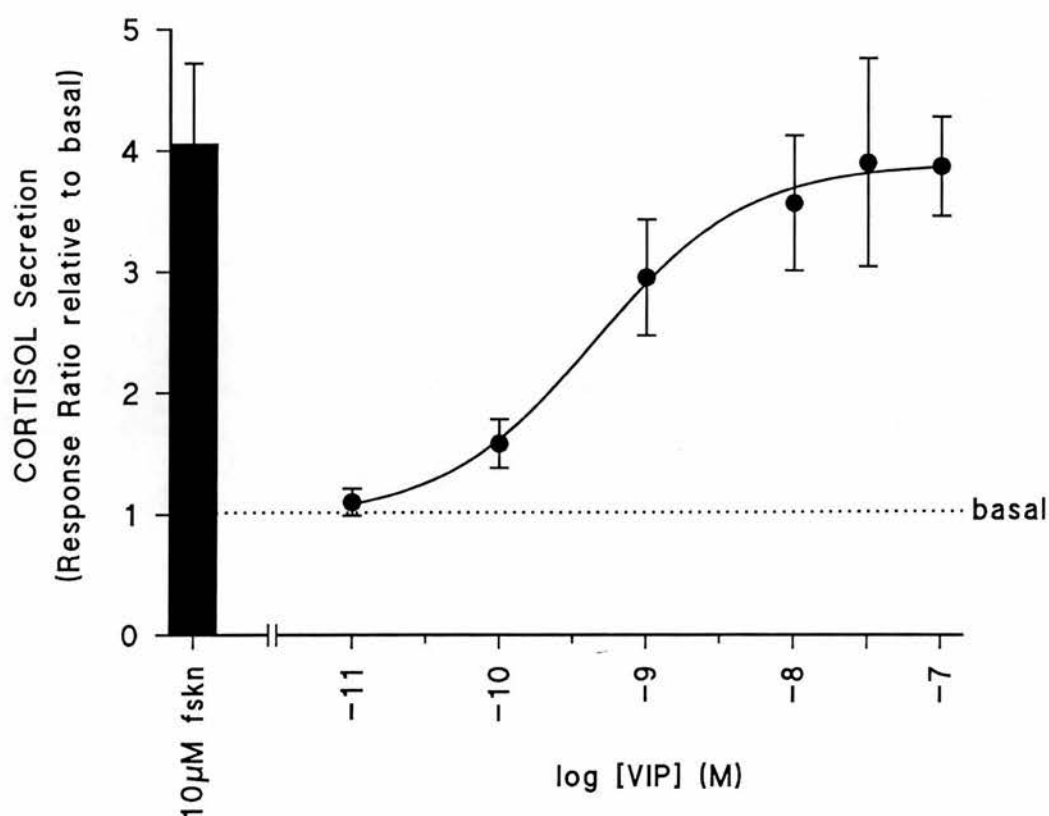


Figure 5.3

Concentration-dependent increase in cortisol secretion in response to 4 h treatment with VIP. Forskolin-pretreated cells were washed and then treated with VIP (10^{-11} M to 10^{-7} M, ●) or forskolin (10^{-5} M, solid bar labelled fskn) for 4 h. After this time, medium was removed and assayed for cortisol content by RIA. Cortisol data was corrected for total cellular protein content. Data from 4 separate experiments, triplicate incubations performed in each, were combined and expressed as mean (\pm s.d.) Response Ratios (response to stimulus / basal secretion).

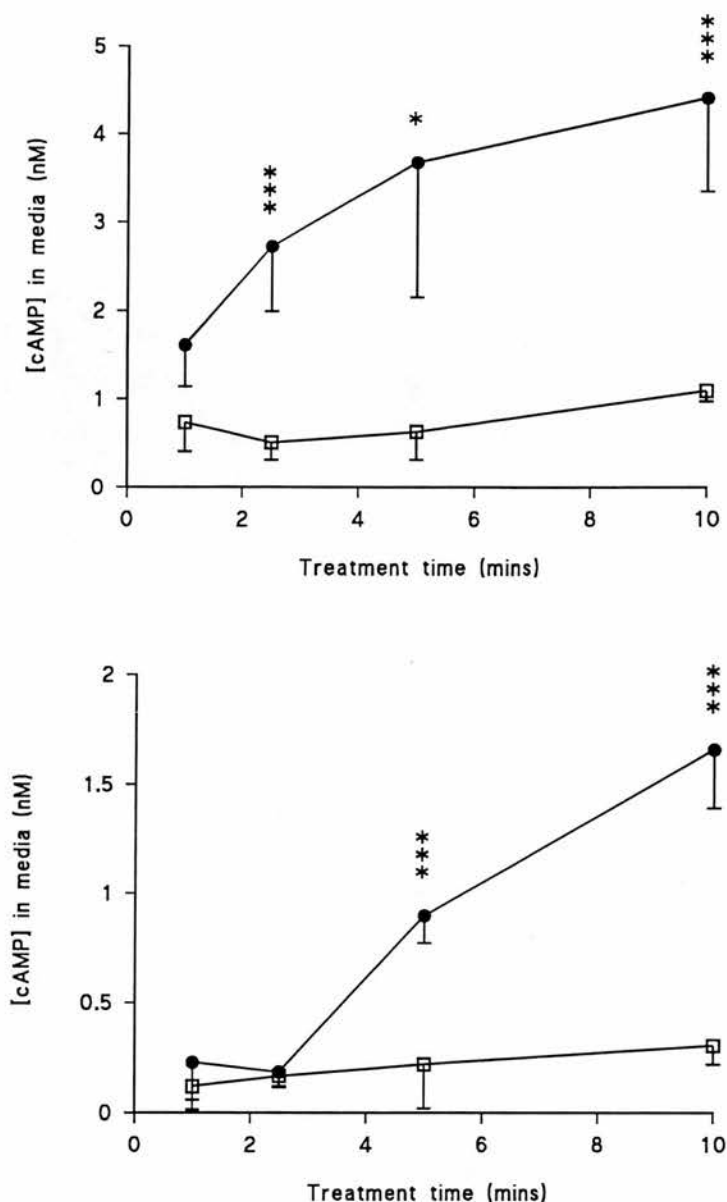


Figure 5.4a

*Time-dependent accumulation of cAMP in medium following treatment with VIP (1-10 mins). Cells were treated with serum-free growth medium alone (basal, □) or containing VIP (3.3×10^{-8} M, ●) for 1, 2.5, 5, 10, 20, 60, 120 or 240 mins. After the defined treatment period, medium was collected and processed (as described in Methods) for analysis of cAMP content by RIA. Results presented are mean (\pm s.d.) for triplicate incubations from 2 separate experiments. Figure 5.4a shows the time-points from 1 to 10 mins. Figure 5.4b (next page) shows the entire time-course from 1 to 240 mins. A statistically significant difference between basal cAMP concentration and VIP-stimulated cAMP levels at any particular time-point is indicated by *P<0.05 and ***P<0.01.*

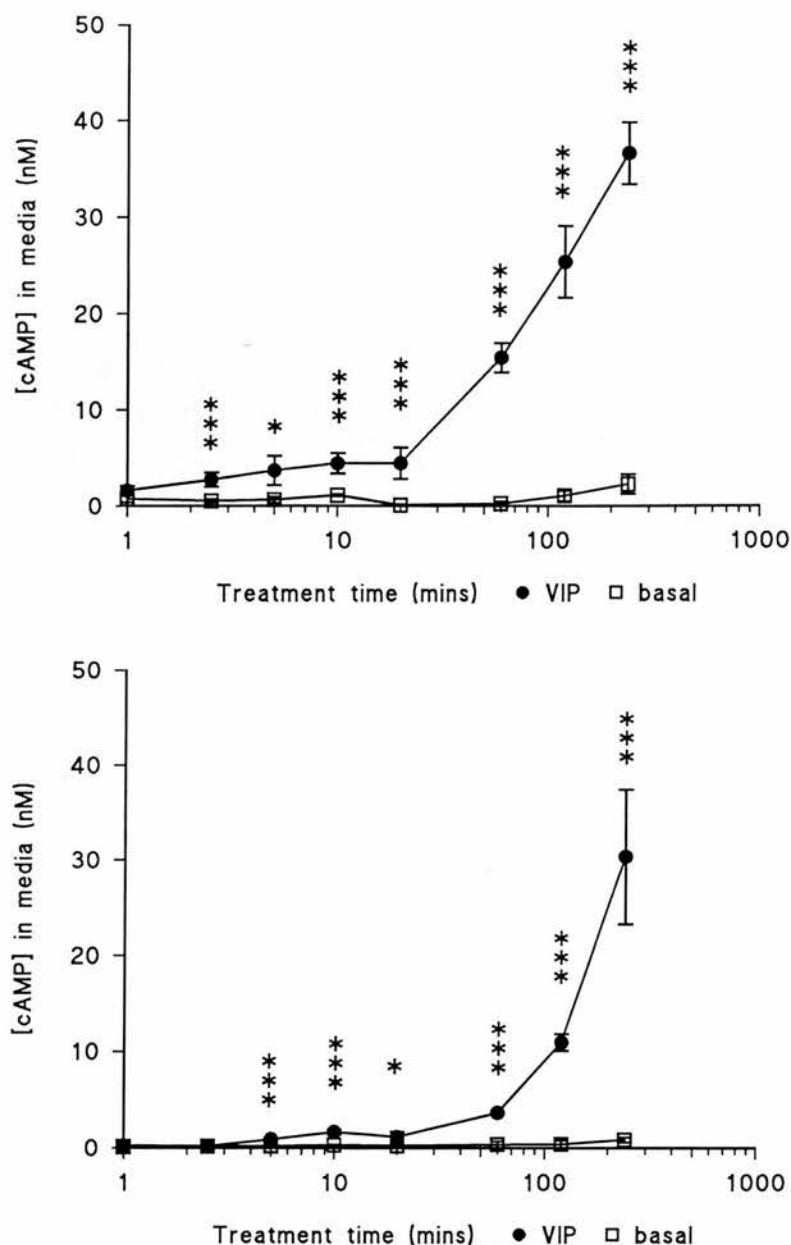


Figure 5.4b

Time-dependent accumulation of cAMP in medium following treatment with VIP (1-240 mins). Cells were treated with serum-free growth medium alone (basal, □) or containing VIP (3.3×10^{-8} M, ●) for 1, 2.5, 5, 10, 20, 60, 120 or 240 mins. After the defined treatment period, medium was collected and processed (as described in Methods) for analysis of cAMP content by RIA. Results presented are mean (\pm s.d.) for triplicate incubations from 2 separate experiments. Figure 5.4a (previous page) shows the time-points from 1 to 10 mins. Figure 5.4b shows the cAMP accumulation for each time-point over the time-course from 1 to 240 mins. A statistically significant difference between basal cAMP concentration and VIP-stimulated cAMP levels at any particular time-point is indicated by *P<0.05 and ***P<0.01.

with VIP for the cortisol studies, a time-point of 4 h was chosen for measurement of cAMP accumulation in response to different concentrations of VIP.

(b) cAMP concentration-response curves to VIP

Figure 5.5(A) shows the concentration-dependent increase in cAMP accumulation following VIP treatment, reaching a maximum of 16 (± 11)-fold increase with 3.3×10^{-8} M (from $n=4$ individual experiments with n -fold increases of 10.0, 10.8, 32.6 and 11.0), with a threshold change occurring at 10^{-11} M ($n=4$ individual experiments). The maximum cAMP response was similar to that induced by 10 μ M forskolin. *Figure 5.5(B)* shows the cortisol concentration-response curve for comparison.

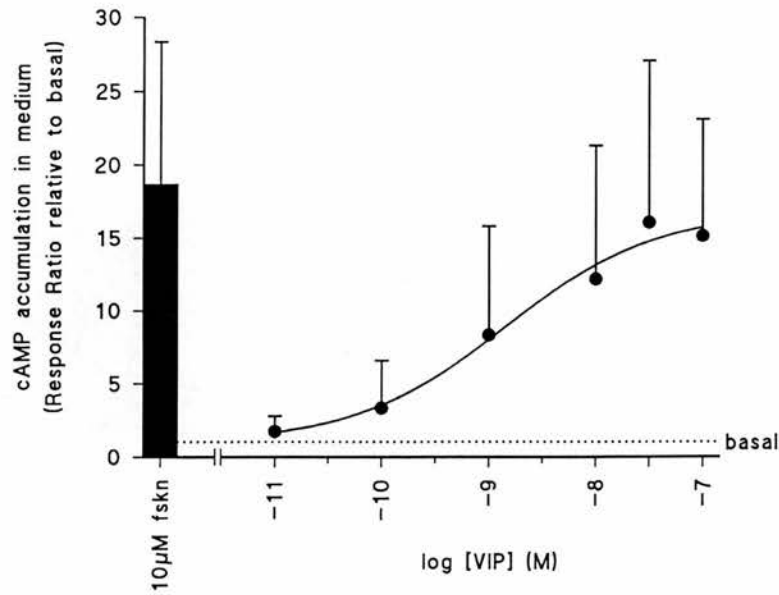
The mean pEC₅₀ (\pm s.d.) obtained for cAMP accumulation in the media was 8.6 (± 0.45) ($n=4$ experiments). *Table 5.1* shows the individual pEC₅₀ values obtained for cortisol and cAMP production from 4 separate experiments in response to VIP. The mean pEC₅₀ values for cortisol and cAMP production were not statistically different ($P \geq 0.05$).

5.2.2.2 Measurement of cGMP production following treatment of H295R cells with VIP

(a) Time course and concentration-response investigations

Time-courses, using a concentration of 3.3×10^{-8} M VIP, and concentration-response curves to VIP (4 h treatment) were performed. In some experiments, there appeared to be an increase in cGMP over basal levels (see *Table 5.2*). However, the levels of cGMP detected in most of these experiments were very low and two concerns presented themselves, which were investigated: 1) was the apparent increase in cGMP actually a result of cross-reactivity of the cGMP antibody with the high levels of cAMP produced in response to VIP? and 2) were the extremely low levels detected greater than the minimum detection limit for the assay?

(A)



(B)

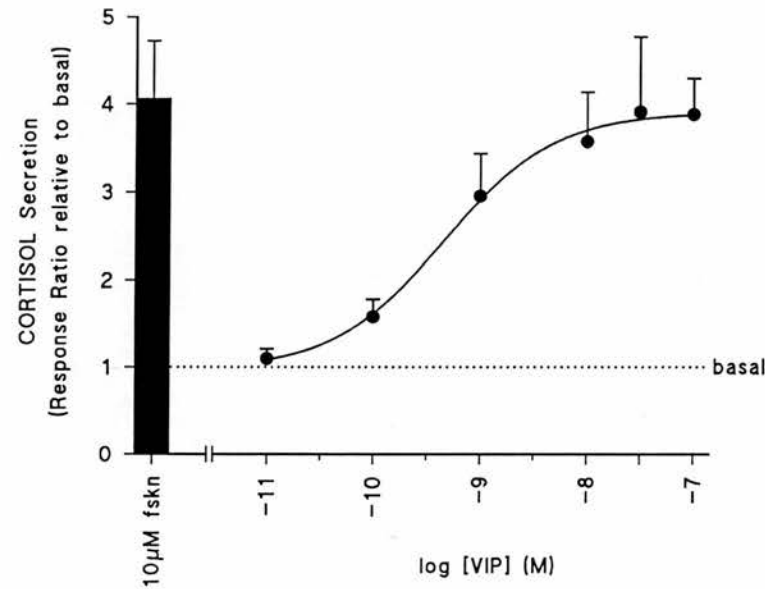


Figure 5.5

Concentration-dependent increase in cAMP secretion into the medium in response to 4 h treatment with VIP (A). Cortisol secretion from identically-treated H295R cells is shown in (B) for comparison. Forskolin-pretreated cells were washed thoroughly, then treated with VIP (10^{-11} to 10^{-7} M, ●) or forskolin (10^{-5} M, solid bar) for 4 h. Aliquots of medium were assayed directly for cortisol content by RIA or processed (as described in Methods) for cAMP measurement by RIA. Data (corrected for total cellular protein) was combined and is expressed as mean (\pm s.d.) Response Ratios (response to stimulus / basal secretion, RR) from 4 separate experiments for both cortisol and cAMP results.

pEC50 (+/-s.d.) for VIP-induced responses		
	CORTISOL response	cAMP response
<i>Expt.1</i>	9.533 (0.10)	9.193 (0.10)
<i>Expt.2</i>	8.921 (0.12)	8.294 (0.33)
<i>Expt.3</i>	9.640 (0.19)	8.591 (0.21)
<i>Expt.4</i>	8.796 (0.14)	8.194 (0.21)
Mean pEC50 (+/-s.d.)	9.223 (0.43)	8.568 (0.45)

Table 5.1

Comparison of pEC50 values for cortisol secretion and cAMP production from H295R cells treated with a range of doses of VIP (10^{-11} M - 10^{-7} M) for 4 h. Treatment of cells, processing of medium and analysis of medium content of cortisol and cAMP by specific RIAs was carried out as described in Methods. Data expressed are mean (\pm s.d.) pEC50 from triplicate incubations performed in each of n=4 experiments. The mean (\pm s.d.) pEC50 values from the combined data are also expressed.

cGMP response to VIP (pmol / mg protein / 4 h)					
	<i>Expt. 1</i>	<i>Expt. 2</i>	<i>Expt. 3</i>	mean	s.d.
<i>basal</i>	0.08	0.23	0.24	0.18	0.09
<i>VIP 10⁻¹¹ M</i>	0.17	0.25	0.24	0.22	0.04
<i>VIP 10⁻¹⁰ M</i>	0.24	0.30	0.37	0.30	0.07
<i>VIP 10⁻⁹ M</i>	0.30	0.41	0.42	0.38	0.07
<i>VIP 10⁻⁸ M</i>	0.39	0.41	0.41	0.40	0.01
<i>VIP 3.3 x 10⁻⁸ M</i>	0.39	0.43	0.50	0.44	0.06
<i>VIP 10⁻⁷ M</i>	0.39	0.54	0.41	0.45	0.08

Table 5.2

Measurement of cGMP levels in overlying medium of H295R cells following 4 h treatment with VIP. Forskolin-pretreated cells were washed thoroughly and then treated with serum-free medium alone (basal) or containing VIP (10^{-11} M - 10^{-7} M) for 4 h. After this time, medium was collected and processed (as described in Methods) and assayed for cGMP content by RIA. Data from n=3 separate experiments, triplicate incubations performed in each, were corrected for total cellular protein and are presented in *Table 5.2* with the mean (\pm s.d.) of combined data.

(b) Calculation of cross-reactivity of cAMP with cGMP antibody

The cross-reactivity of the cGMP antibody with cAMP was determined by analysing the binding of acetylated cAMP standards (1 μ M to 1 mM) to the cGMP antibody in comparison to acetylated cGMP standards. The logit B/B₀ was plotted against the cyclic nucleotide concentration on a logarithmic scale. This produced a linear function. Cross-reactivity was estimated at logit B/B₀ = 0. *Figure 5.6* shows this plot. The cross-reactivity, determined from 3 separate experiments was 0.01 (± 0.003) %. Based on this level of cross-reactivity, the cAMP produced in response to VIP is unlikely to account for the cGMP detected.

(c) Calculation of minimum detection limit (MDL) of cGMP RIA

The MDL for the assay was calculated by combining and plotting the precision profile data from n=9 assays. *Figure 5.7* shows the graph of the precision profile from which the MDL was calculated by reading the concentration of cGMP at which there is a 22% CV (McConway *et al*, 1989). The MDL was calculated to be ≥ 0.25 nM. Most of the cGMP levels measured from the various experiments performed were below this limit. Inclusion of the phosphodiesterase inhibitor, IBMX, in the experimental media did not alter the cyclic nucleotide concentrations in these cells.

5.2.2.3 Measurement of phosphoinositide turnover in response to VIP

VIP (3.3×10^{-8} M) failed to increase phosphoinositide turnover in H295R cells (n=3 experiments, $P \geq 0.05$, calculated from triplicate incubations within each experiment), whereas the positive control treatment, AII (10^{-8} M), caused a 2.4 (± 0.25)-fold increase in phosphoinositide turnover from “control” pretreated cells (n=3 experiments, $P < 0.02$ for 2/3 experiments, calculated from triplicate incubations within each experiment) and a 1.5 (± 0.15)-fold increase in forskolin-pretreated cells

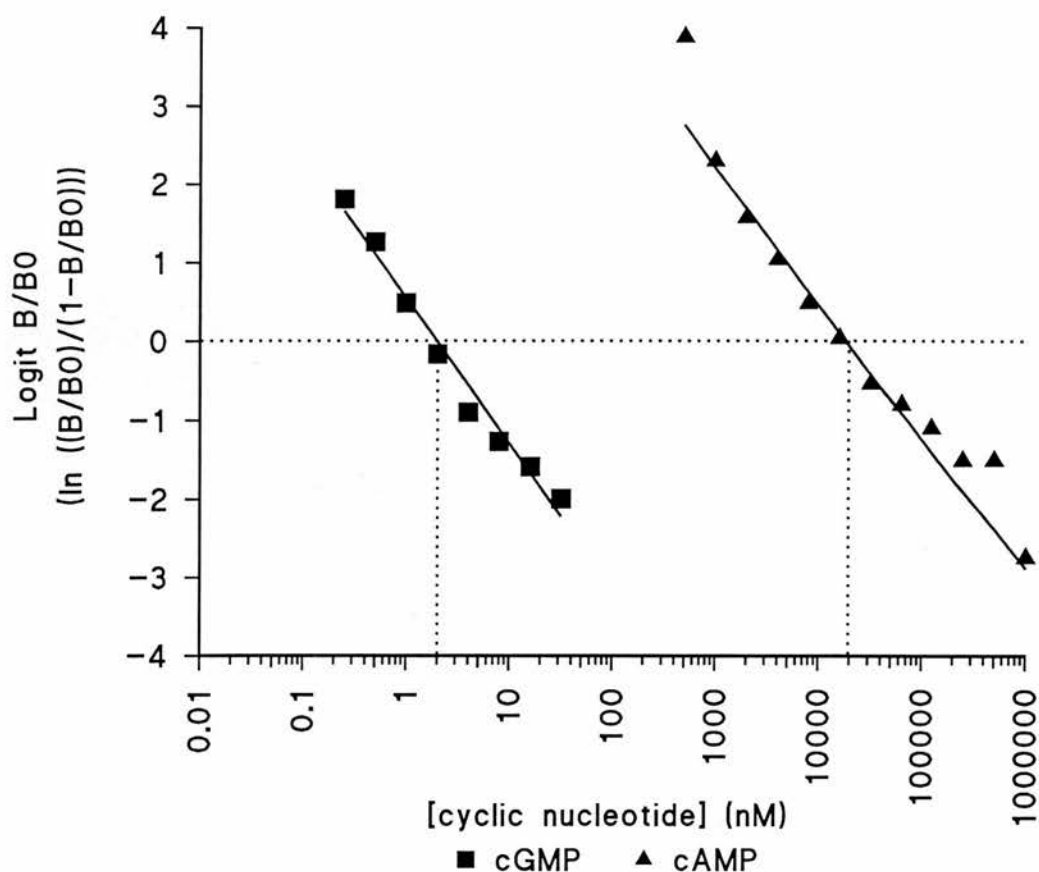


Figure 5.6

Calculation of cAMP cross-reactivity with the cGMP antibody. cAMP standards ▲ (1 μ M-1 mM) were prepared in the same way as the usual cGMP standards ■ (0.03 nM-32 nM) used for the RIA standard curve. The assay was performed as normal, with the cAMP standards measured as samples. B/B0 was calculated for each standard (cGMP and cAMP) and the logit function plotted against the cyclic nucleotide concentration on a logarithmic scale. The cross-reactivity of cAMP with the cGMP antibody was calculated at the 0 logit point. This graph is one of a set of n=3 experiments performed.

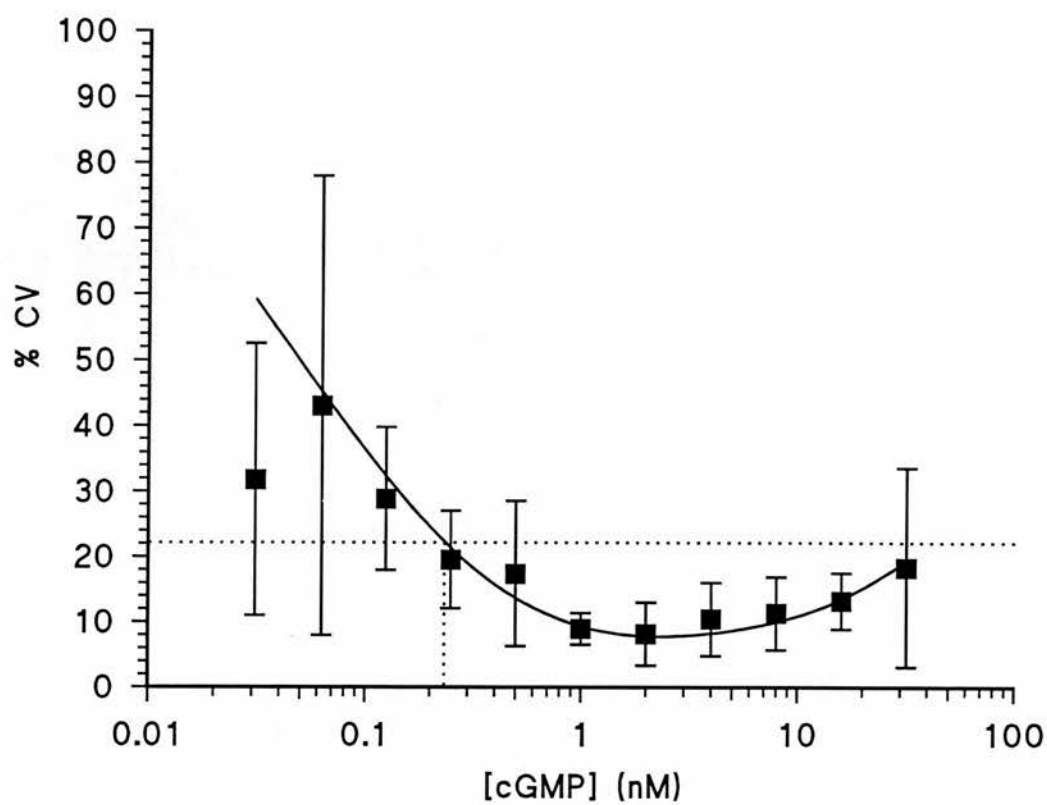


Figure 5.7

Calculation of the minimum detection limit for the cGMP RIA employed. Precision profile data from $n=9$ separate assays were combined and the dose-error curve plotted, as shown above. The minimum detection limit was taken as the concentration of cGMP with a CV (coefficient of variation) equal to 22% (McConway *et al*, 1989).

(a)

[³ H]-phosphoinositol accumulation in response to VIP-control cells (dpm)			
	expt 1	expt 2	expt 3
basal	1671 (799)	2481 (821)	3843 (229)
VIP 10 ⁻¹¹ M	1239 (123)	2373 (589)	4297 (318)
VIP 10 ⁻¹⁰ M	1050 (255)	3022 (97)	3201 (194)
VIP 10 ⁻⁹ M	1448 (368)	3166 (40)	3828 (195)
VIP 10 ⁻⁸ M	1126 (92)	3349 (385)	3831 (36)
VIP 3.3 x 10 ⁻⁸ M	1630 (108)	3544 (282)	3677 (77)
All 10 ⁻⁸ M	3580 (111)	**5710 (432)	***10109 (613)

(b)

[³ H]-phosphoinositol accumulation in response to VIP-forskolin pretreated cells (dpm)			
	expt 1	expt 2	expt 3
basal	2905 (559)	5570 (508)	5793 (314)
VIP 10 ⁻¹¹ M	2922 (430)	5373 (216)	6522 (219)
VIP 10 ⁻¹⁰ M	3165 (474)	5535 (581)	7653 (1649)
VIP 10 ⁻⁹ M	2959 (567)	5141 (174)	7115 (860)
VIP 10 ⁻⁸ M	3221 (203)	5655 (240)	6485 (329)
VIP 3.3 x 10 ⁻⁸ M	3228 (74)	6592 (500)	6502 (982)
All 10 ⁻⁸ M	4518 (281)	7400 (1045)	***9413 (445)

Table 5.3

Effect of VIP and AII on phosphoinositide breakdown in (a) control pretreated and (b) forskolin pretreated H295R cells. Cells were pretreated for 72 h with either growth medium alone (a) or forskolin (10 μ M) (b). During the last 48 h of this pretreatment period cells were incubated with [³H]-inositol to label membrane inositol phospholipids. Cells were stimulated in DMEM / F12 without agonists (basal) or with various doses of VIP (10⁻¹¹ M - 3.3 x 10⁻⁸ M) or AII (10⁻⁸ M) for 30 mins. Phosphoinositide breakdown was assayed as described in Methods. Data presented here are from n=3 experiments, with triplicate incubations performed in each., and are expressed as mean disintegrations per minute (dpm) (\pm s.d.). Statistically significant changes in dpm measured with respect to basal are represented by **P<0.02 and ***P<0.01.

(n=3 experiments, $P < 0.01$ in 1/3 experiments, calculated from triplicate incubations within each experiment) (see *Table 5.3*)

5.3 Discussion

The aim of this chapter was to determine the second messenger responses elicited by VIP in H295R cells.

In other tissues, VIP stimulation has been linked to the activation of a number of second messenger systems, including cAMP production, cGMP production, increased phosphoinositide turnover and changes in intracellular Ca^{2+} levels (section 5.1.4). In the adrenal gland, stimulation of steroidogenesis by the physiological regulators, ACTH and AII has been predominantly associated with increased cAMP formation and phosphoinositide turnover and calcium influx, respectively (sections 3.1.1 and 3.1.2).

VIP increased cortisol secretion from H295R cells in a concentration-dependent manner. Maximal stimulation occurred at a concentration of 3.3×10^{-8} M VIP, which was similar to that observed for glucocorticoid secretion from dispersed rat adrenocortical cells (Mazzocchi *et al*, 1994b; maximal stimulation of cortisol secretion observed with a concentration of 10^{-8} M), perfused porcine adrenal glands (Ehrhart-Bornstein *et al*, 1991a; maximal stimulation with 10^{-8} M VIP) and cultured human adrenal cells (Bornstein *et al*, 1996; maximal stimulation with 10^{-7} M). The maximal cortisol response to VIP from H295R cells was equivalent to that produced by 10 μM forskolin, indicating that VIP is a potent stimulator of cortisol production in these cells.

The cAMP concentration-response curve paralleled the cortisol concentration-response curve in that maximal cAMP production occurred at the same concentration of VIP as maximal cortisol secretion (3.3×10^{-8} M). The threshold concentration of VIP was also the same for cortisol and cAMP and the pEC₅₀ values for cortisol and cAMP production were not significantly different (9.22 ± 0.43 compared with 8.7 ± 0.45 , respectively, $P \geq 0.05$). In addition, 3.3×10^{-8} M VIP was equipotent with 10 μM forskolin for cortisol secretion and similar in potency to forskolin-stimulated cAMP production. Bodart *et al* (1997) recently investigated the effect of VIP on human adrenocortical tumour cells, with respect to aldosterone

secretion, and demonstrated a potent effect on cAMP production with a similar EC₅₀ (0.38 ± 0.021 nM) to that obtained in this study.

The stimulation of cGMP production in adrenocortical cells has been associated with an inhibitory effect on steroidogenesis since ANP inhibits aldosterone production and also causes an increase in cGMP (Ganguly, 1992, and section 7.1.1). However, the mechanism of cGMP action in adrenocortical cells is still unclear. A few groups have implicated cGMP as a mediator of ACTH-stimulated steroidogenesis in adrenal zf cells (Harrington *et al*, 1978; Perchellet & Sharma, 1979), though the increase was transient (Perchellet & Sharma, 1979) and was often not reproducible.

VIP has been shown to stimulate an increase in cGMP levels, in conjunction with an increase in cAMP, in rat pinealocytes (section 5.1.4). The possibility that VIP activates a similar dual signalling system in adrenocortical cells was investigated. There was no convincing cGMP response within the limits of the RIA employed. Inclusion of the phosphodiesterase inhibitor, IBMX, in the experimental media did not alter the amount of either cyclic nucleotide detected. This suggests that cyclic nucleotide phosphodiesterase activity is intrinsically low in these cells. Further investigation of a possible VIP-induced effect on cGMP levels in these cells would be worth pursuing in future experiments.

Turnover of phosphoinositides was investigated in response to VIP and AII. AII is well documented as a stimulant for phosphoinositide turnover leading to Ins(1,4,5)P₃ production. VIP has been shown to affect components of this intracellular signalling system in some tissues (section 5.1.4), though it is unclear whether these effects are mediated by VIP receptors (VPAC / PACAP type II receptors) or via PACAP-specific receptors (PAC1 / PACAP type I receptors). Indeed, phosphoinositide turnover in rat superior cervical ganglia required much higher concentrations of VIP than were needed for cAMP production in the same tissue (Audigier *et al*, 1986). VIP is capable of activating PACAP type I (PAC1) receptors but with a greatly reduced potency (section 6.1). VIP had no effect on phosphoinositide turnover in H295R cells whereas AII stimulated a small but

significant increase, consistent with its weak agonist action in our H295R cells (chapter 3.0).

Thus, there was no convincing cGMP response to VIP from H295R cells and VIP also failed to increase phosphoinositide turnover, whereas cAMP appears to be the major intracellular messenger increased by VIP in these cells. It can be concluded that VIP regulates steroidogenesis from H295R cells by a cAMP-dependent mechanism, similar to ACTH. This is consistent with the observation that the main steroid secreted in response to VIP from this cell-line was cortisol (see chapter 4.0). Ehrhart-Bornstein *et al* (1991a) also detected a greater cortisol response than aldosterone response to VIP from perfused, porcine adrenals.

Recently, Mazzocchi *et al* (1998) demonstrated VIP-stimulated glucocorticoid secretion and cAMP production from dispersed rat inner zone adrenocortical cells. Because the effect was anulled by both a VIP-receptor antagonist ([4-Cl-D-Phe⁶,Leu¹⁷]-VIP) and an ACTH-receptor antagonist (corticotropin-inhibiting peptide, CIP), they attributed the response to an activation of ACTH receptors by VIP. This was in accordance with previous work from this group which indicated that VIP increased basal, but not submaximally ACTH-stimulated corticosterone secretion from dispersed rat inner zone cells (Mazzocchi *et al*, 1994b). The VIP-receptor antagonist ([4-Cl-D-Phe⁶,Leu¹⁷]-VIP) partially abolished the ACTH-mediated steroid response and CIP completely abolished it (Mazzocchi *et al*, 1994b). Li *et al* (1990) reported that VIP competes for a subtype of ACTH receptor; those recognising the ACTH₁₁₋₂₄ sequence. However, VIP competitively abolished radiolabelled VIP binding in the rat adrenal cortex and ACTH was only capable of competing for a subset of these receptors (Cunningham & Holzwarth, 1989). The effect of VIP on cortisol secretion from H295R cells is unlikely to involve ACTH receptor activation because the steroid and cAMP responses to ACTH from these cells were poor when compared with responses to forskolin (Rainey *et al*, 1993). Indeed, maximal stimulation of cAMP production, P450c17 activity and cortisol secretion by forskolin (7-20 μ M) was approximately 7, 5 and 4 times greater, respectively, than the maximum responses to ACTH (0.1 μ M)

(Rainey *et al*, 1993). In the experiments described in this chapter, maximal stimulation of cAMP and cortisol by VIP was similar to that produced by forskolin (10 μ M). It has been suggested that the ACTH receptor number on these cells is low, a theory supported by the finding that, although levels of ACTH receptor mRNA in this cell-line can be upregulated by cAMP agonists, ACTH was much less potent than forskolin or dbcAMP at eliciting this effect (Mountjoy *et al*, 1994). Alternatively, coupling between the ACTH receptor and the adenylate cyclase-cAMP pathway may be defective in these cells. Thus, the VIP-induced steroid response from this human adrenocortical cell-line is unlikely to involve ACTH receptor activation. A species-specific difference may explain the findings of Mazzocchi *et al* (1998). Indeed, Haidan *et al* (1996) reported that the effects of VIP and ACTH on steroid secretion from dispersed human adrenocortical cells were additive. In addition, Bodart *et al* (1997) indicated a species-specific difference when comparing the effects of VIP on human adrenocortical carcinoma cells and bovine zg cells. Although the involvement of ACTH receptors in the VIP-induced steroid response *in vivo* cannot be excluded in man, the findings in this chapter indicate that VIP-induced steroid responses in the human adrenal gland involve a non-ACTH dependent mechanism.

Controversy surrounding a direct action for VIP on adrenocortical cells arose because some authors were unable to detect a VIP-stimulated steroid response from dispersed adrenocortical cells. Most of these studies were performed on dispersed zg cells (Enyedi *et al*, 1983; Hinson *et al*, 1992; Bodart *et al*, 1997). However, experiments performed on bovine and rat dispersed inner zone adrenocortical cells have demonstrated a glucocorticoid response to VIP (Li *et al*, 1990; Mazzocchi *et al*, 1994b). Whilst an indirect, catecholamine-mediated action of VIP is undisputed, the hypothesis may be advanced that a direct steroid action of VIP is concentrated in the zfr, whilst the indirect mechanism may be predominant in the zg. This would be in keeping with the ACTH-like action of VIP in H295R cells in that cortisol was the major steroid secreted and that cAMP was the only significant second messenger response elicited following VIP treatment of the cells.

Although autoradiographic binding studies for VIP receptors in the rat showed a predominance of VIP receptors in the zg region of the adrenal gland (Cunningham & Holzwarth, 1989), a species-specific difference between rats and humans may exist. A direct steroidogenic action of VIP in the zg is not ruled out but may be less significant than in the zfr. Recently, Hinson *et al* (1996) demonstrated an increase in VIP-induced steroid response and VIP binding in the zg following salt deprivation of rats. Thus the direct action of VIP in the zg is capable of upregulation in certain circumstances, perhaps providing an extra layer for control of steroid regulation during changes in electrolyte status. A direct action of VIP on zg cells could involve growth effects since administration of a VIP receptor antagonist annulled VIP-induced growth of the zg in rats (with pharmacologically interrupted HPA axis and RAS) and from zg-like cells of rat adrenocortical autotransplants (Rebuffat *et al*, 1994).

In addition, though VIP receptor number may be lower in the zfr, the number of gap junctions present between zfr cells of rodent species has been shown to be greater than in the zg (Murray & Pharrams, 1997), such that stimulation of a VIP receptor on one cell may result in activation of a steroid response in several surrounding cells. Gap junctions provide pathways for direct intercellular movement of small molecules, including nucleotides (Pitts *et al*, 1971). In particular, the involvement of gap junctions was important in increasing steroid responsiveness of bovine and human zfr cells to low concentrations of ACTH (Munarilem *et al*, 1995). Bovine adrenals exhibited a similar distribution of gap junctions in the zg and the zfr, in contrast with rodent adrenals, though the gap junctions present in each zone were numerous and large (Murray & Pharrams, 1997). Also, more than one subtype of VIP receptor exists. The second messenger coupling of receptors in the zfr may be better than in the zg, thus producing more cAMP for VIP-stimulated steroidogenesis.

Gallo-Payet & Escher (1985) demonstrated a higher number of ACTH receptors on rat zg cells than zfr cells, yet ACTH produced a higher increment in steroid output from zfr cells than from zg cells (Braley & Williams, 1977). Levels of

phosphodiesterase are also higher in rat zg cells compared with zfr cells (Gallant *et al*, 1974). Thus, a direct action of VIP involving cAMP production could result in a less pronounced effect of VIP in zg cells, due to more rapid cyclic nucleotide degradation by phosphodiesterase, despite the presence of more receptors. Also, the presence of spare receptors for ACTH in the adrenal has been suggested (Hornsby & Gill, 1981). The phenomenon of spare receptors means that not all receptors need to be activated to obtain a maximal response. Thus, lower numbers of VIP receptors within the zfr, compared to the zg, do not necessarily equate to a lower potency of VIP or a lesser steroid response.

In summary, VIP stimulated a concentration-dependent increase in cortisol secretion from H295R cells which was accompanied by a concentration-dependent rise in cAMP accumulation. At this stage one has to conclude that cGMP levels were unaffected by VIP treatment and similarly a phosphoinositide response to VIP was absent in H295R cells.

6.0 Characterisation of receptor types activated by VIP in H295R cells

6.1 Introduction

In earlier chapters it was observed that VIP stimulates steroid synthesis from H295R cells (chapter 4.0) via a mechanism involving increased accumulation of cAMP (chapter 5.0). The steroidogenic action of VIP could involve activation of a number of different receptors. Possible mechanisms include: 1) a mechanism involving β -adrenoceptors, 2) direct activation of a VIP receptor (currently comprising the VPAC1 receptor or VPAC2 receptor subtypes), 3) direct activation of the pituitary adenylate cyclase activating polypeptide (PACAP) type I receptor (PAC1 receptor). The VIP / PACAP receptor family is introduced in section 6.1.1. The nomenclature used to refer to these receptors is that suggested by the International Union of Pharmacology (Harmar *et al*, 1998).

6.1.1 The VIP / PACAP receptor family

Receptors for VIP belong to a subfamily of the seven transmembrane, G-protein-linked receptor superfamily (Segre & Goldring, 1993). This subfamily of receptors is distinct (showing < 12 % sequence identity) from other G-protein-linked receptors such as rhodopsin. Other receptors in this subfamily include those for the following peptides: secretin, calcitonin, parathyroid hormone, parathyroid hormone-related peptide, glucagon, glucagon-like peptide 1, growth hormone-releasing hormone and PACAP. Due to the high homology between some of these peptides (*Figure 1.7*) and also between receptors in the subfamily, many of these peptides are capable of activating other receptors within the same receptor subfamily. In particular, VIP and PACAP share a receptor, the VPAC receptor, for which they have a similar affinity and potency. Two subtypes of the VPAC receptor have been identified: the VPAC1 receptor and the VPAC2 receptor (sections 6.1.1.1 and 6.1.1.2). Initial indications of these two receptor types came from pharmacological studies in different tissues and cell-lines (Laburthe *et al*, 1983; Robberecht *et al*,

1988; Luis & Said, 1990) and have since been confirmed by molecular cloning of the two receptor types and pharmacological characterisation in transfected cell-lines. Both receptors are bound and activated by PACAP38 and PACAP27 with a similar potency to VIP (Svoboda *et al*, 1994; Lutz *et al*, 1993; Usdin *et al*, 1994; Ishihara *et al*, 1992; Sreedharan *et al*, 1993) (Table 6.1). However, the VPAC2 receptor differs pharmacologically from the VPAC1 receptor in that 1) helodermin stimulates cAMP production with similar potency to VIP (hence, the VPAC2 receptor has been suggested to be the helodermin-preferring receptor) (Adamou *et al*, 1995; Lutz *et al*, 1993; Svoboda *et al*, 1994), whereas helodermin was less potent than VIP on the VPAC1 receptor (Ishihara *et al*, 1992; Gaudin *et al*, 1996) and 2) secretin is relatively ineffective at eliciting a cAMP response from the VPAC2 receptor (Adamou *et al*, 1995; Lutz *et al*, 1993; Svoboda *et al*, 1994; Usdin *et al*, 1994), but activates the VPAC1 receptor with approximately 10-100 times less potency than VIP (Ishihara *et al*, 1992; Sreedharan *et al*, 1993; Usdin *et al*, 1994).

So far, all members of this receptor subfamily have been found to be coupled to a G_s protein with subsequent activation of adenylate cyclase and increased cAMP production. Coupling to other G-proteins has also been observed for some members, for example the PAC1 receptor. Other common features between members of this subfamily include long amino-terminal extensions with a highly conserved pattern of cysteine residues and highly conserved sequences for the V, VI and VII membrane-spanning domains and carboxy-terminal tail (Segre & Goldring, 1993).

6.1.1.1 The VPAC1 receptor

Originally referred to as the VIP receptor, the VIP1 receptor (Lutz *et al*, 1993), the VIP / PACAP type II receptor (Ciccarelli *et al*, 1994) or the PVR2 receptor (Rawlings *et al*, 1995), the VPAC1 receptor (Harmar *et al*, 1998) was first isolated and cloned from rat lung by Ishihara *et al* (1992) using the rat secretin receptor cDNA as a probe. The rat VPAC1 receptor was found to contain seven transmembrane segments and to consist of 459 amino acids with a calculated molecular weight of approximately 52 kDa (Ishihara *et al*, 1992). The receptor had 48 % sequence identity with the rat secretin receptor and mediated VIP-stimulated

Relative binding potencies for VIP / PACAP receptors	
PAC1a receptor:	PACAP38 \approx PACAP27 >>> VIP
PAC1b receptor:	PACAP38 > PACAP27 >>> VIP
VPAC1 receptor:	VIP \approx PACAP27 \geq PACAP38
VPAC2 receptor:	VIP \approx PACAP38 \approx PACAP27

Table 6.1

Relative binding potencies of VIP, PACAP38 and PACAP27 on the PAC1 (a and b), VPAC1 and VPAC2 receptors.

Relevant references include: Svoboda *et al*, 1994; Lutz *et al*, 1993; Usdin *et al*, 1994; Ishihara *et al*, 1992; Sreedharan *et al*, 1993; Van Rampelbergh *et al*, 1996.

accumulation of intracellular cAMP when expressed in COSGs1 cells (Ishihara *et al*, 1992).

The human VPAC1 receptor was cloned from the HT29 intestinal epithelial cell-line (Sreedharan *et al*, 1993) and from jejunal epithelial cells (Couvineau *et al*, 1994) and comprises a 457 amino acid (Sreedharan *et al*, 1993) / 460 amino acid (Couvineau *et al*, 1994) receptor with an estimated molecular mass of approximately 52 kDa. The sequences for both the rat and human VPAC1 receptor appear to encode a signal peptide (Couvineau *et al*, 1994) giving the mature receptor proteins for both species a predicted molecular weight of approximately 49 kDa (Couvineau *et al*, 1994; Ishihara *et al*, 1992). The human VPAC1 receptor gene contains 13 exons and 12 introns and has been localised to the short arm of human chromosome 3 (3p22) (Sreedharan *et al*, 1995).

The amino acid sequence for the human VPAC1 receptor shows 84 % homology with the rat lung VPAC1 receptor (Sreedharan *et al*, 1993). The human sequence contains four potential N-linked glycosylation sites (three in the amino terminus and one in the second extracellular loop) (Couvineau *et al*, 1994). The heterogeneity of molecular weights of VIP receptors detected by cross-linking studies, between tissues and between species (from approximately 51.8 kDa to 68.8 kDa) has been attributed to differences in the size and composition of the oligosaccharide chains attached (Fabre *et al*, 1993b). For example, Fabre *et al* (1993a) characterised a 68.2 kDa VIP receptor from a human pancreatic cell-line which contained at least three N-linked oligosaccharide chains and Gaudin *et al* (1996) characterised a 67 kDa VPAC1 receptor identified by cross-linking experiments.

A potential protein kinase A phosphorylation site was predicted in the C-terminal intracellular tail along with three potential sites for protein kinase C phosphorylation (two in the N-terminal extracellular segment and one in the second intracellular loop) (Couvineau *et al*, 1994).

The amino-terminal domain of the VIP receptor is involved in VIP binding (Couvineau *et al*, 1994; Holtmann *et al*, 1995). In particular, site-directed mutagenesis studies have indicated that the residues aspartate 68, tryptophan 73 and

glycine 109 in the N-terminal extracellular domain of the human VIP receptor are essential for VIP binding (Couvineau *et al*, 1995) along with six cysteine residues, at positions 50, 63, 72, 86, 105 and 122, which may form topologically important disulphide bonds (Gaudin *et al*, 1995). The significance of these cysteine residues was confirmed by the finding that the disulphide reducing agent, dithiothreitol, caused a decrease in specific binding of radiolabelled VIP to vascular VIP receptors (Huang & Rorstad, 1989). In addition, a disulphide bond between cysteine 215 and cysteine 285 was found to be important for stabilising the rat VPAC1 receptor in the correct conformation for ligand binding and receptor activation (Knudsen *et al*, 1997).

6.1.1.2 The VPAC2 receptor

Originally referred to as the VIP2 receptor (Lutz *et al*, 1993), the PACAP-3 receptor (Inagaki *et al*, 1994) or PVR3 (Rawlings *et al*, 1995), the VPAC2 receptor (Harmar *et al*, 1998) was first cloned from the rat olfactory bulb by Lutz *et al* (1993). The putative VPAC2 receptor open reading frame was predicted to encode a 437 amino acid protein of molecular mass 49.5 kDa and an amino acid sequence identity with the rat VPAC1 receptor and PAC1 receptor of 50 % (Lutz *et al*, 1993). Potential sites for N-linked glycosylation were observed in the extracellular amino-terminus at residues 57, 87 and 91 (Lutz *et al*, 1993; Usdin *et al*, 1994). As with the VPAC1 receptor, a highly conserved pattern of cysteine residues was observed in the amino-terminal tail (Lutz *et al*, 1993).

The human VPAC2 receptor, originally cloned from SUP-T1 lymphoblasts (Svoboda *et al*, 1994) and human placenta (Adamou *et al*, 1995), is a 438 amino acid receptor with a predicted molecular weight of 49.5 kDa (including the putative signal peptide) (Adamou *et al*, 1995). It contains three potential N-linked glycosylation sites at residues 58, 88 and 92 and demonstrates an 85-86 % sequence identity with the rat VPAC2 receptor at the amino acid level (Adamou *et al*, 1995; Svoboda *et al*, 1994). The human VPAC2 receptor shows 49 % sequence identity with the human VPAC1 receptor and 52 % identity with the human PAC1 receptor (Adamou *et al*, 1995; Svoboda *et al*, 1994).

The VPAC2 receptor gene maps to human chromosomal position 7q36.3 (Mackay *et al*, 1996).

6.1.1.3 Tissue distribution of VIP receptors

A distinct tissue distribution of VPAC1 and VPAC2 receptor expression, illustrated by *in situ* hybridisation, is evident (Usdin *et al*, 1994). VPAC1 receptor transcripts were detected in a number of tissues, using *in situ* hybridisation and Northern blot analysis, including lung, small intestine, liver and placenta, as well as regions of the brain including the cerebral cortex and hippocampus (Ishihara *et al* 1992; Usdin *et al*, 1994; Sreedharan *et al*, 1993).

Using *in situ* hybridisation and northern blot analysis, the presence of VPAC2 receptor mRNA has been detected in areas of the brain and CNS (Lutz *et al*, 1993; Usdin *et al*, 1994), often in regions distinct from those showing expression of VPAC1 receptor mRNA, for example, the suprachiasmatic nucleus and the hypothalamus (Usdin *et al*, 1994). VPAC2 receptor transcripts have also been detected in peripheral tissues, including the stomach, pituitary and pancreas (Usdin *et al*, 1994; Adamou *et al*, 1995).

Usdin *et al* (1994) reported the presence of both VPAC1 and VPAC2 receptor transcripts in the rat adrenal gland using *in situ* hybridisation. VPAC2 receptor mRNA was found predominantly in the adrenal cortex with VPAC1 receptor gene expression predominant in the adrenal medulla.

6.1.1.4 PACAP and the PAC1 receptor

In 1989 a group investigating hypophysiotropic factors regulating the anterior pituitary isolated a 38 amino acid peptide from ovine hypothalamus based on its ability to stimulate cAMP production from anterior pituitary cells (Miyata *et al*, 1989). This peptide was initially named pituitary adenylate cyclase activating polypeptide (PACAP) (Miyata *et al*, 1989) and later amended to PACAP38, following the isolation from ovine hypothalamic extracts of a peptide consisting of

the N-terminal 27 amino acids of PACAP38, named PACAP27 (Miyata *et al*, 1990). The human PACAP gene has also been isolated and cloned (Hosoya *et al*, 1992).

PACAP38 and PACAP27 have been classified as members of the VIP / secretin / GHRH family of peptides based on the high sequence homology with these peptides. Indeed, the 27 N-terminal amino acids of PACAP share 68% homology with VIP (Miyata *et al*, 1989).

PACAP has been detected mainly in areas of the central nervous system, such as the hypothalamus, posterior pituitary and cerebral cortex, but also in peripheral tissues, including the testis, adrenal and gut (Arimura *et al*, 1991; Sundler *et al*, 1992, Masuo *et al*, 1993).

Ligand binding studies and receptor autoradiography indicated the presence of two high affinity binding sites for PACAP (Shivers *et al*, 1991). One of these sites had a much greater affinity for PACAP27 than for VIP and was subsequently named the PACAP type I receptor, later PVR1 (Rawlings *et al*, 1995) or PAC1 receptor (Harmar *et al*, 1998). The other binding site showed equal affinity for PACAP27 and VIP and was named the PACAP type II receptor (Shivers *et al*, 1991), subsequently shown to be two receptors which correspond to the VPAC1 and VPAC2 receptors (Arimura *et al*, 1992).

The PAC1 receptor was first cloned from a rat pancreatic cancer cell-line (Pisegna & Wank, 1993) and from a rat brain cDNA library (Hashimoto *et al*, 1993). The mature receptor protein was calculated as having 476 amino acids, a predicted molecular weight of 54.4 kDa and five potential N-linked glycosylation sites (Hashimoto *et al*, 1993). So far, a *short* form of the rat PAC1 receptor has been identified along with five different splice variants, dependent on whether they contain either or both of two 84 bp inserts, named *hip* or *hop*, of which two *hop* forms exist; a 28 amino acid insert called *hop1* and a 27 amino acid insert, which lacks a serine residue in the *hop1* sequence, called *hop2* (Spengler *et al*, 1993). The splice variants differ in their intracellular signal transduction mechanisms: The *short* form and the *hop1* and *hop2* forms are linked to activation of adenylate cyclase and phospholipase C, the *hip* form stimulates adenylate cyclase with approximately 10-fold lower potency and has no effect on phospholipase C, whilst the *hiphop1* and

hiphop2 forms possess intermediate characteristics (for review see Journot *et al*, 1995). More recently, another PAC1 receptor splice variant has been discovered in the rat (Pantaloni *et al*, 1996). This variant, the *very short* (*vs*) form, is produced from alternative splicing in the N-terminal extracellular domain and has been shown to modulate receptor selectivity with respect to PACAP27 and PACAP38 binding and relative potencies for PLC stimulation (Pantaloni *et al*, 1996).

The human PAC1 receptor was first isolated from a human pituitary cDNA library (Ogi *et al*, 1993). The mature receptor protein was calculated to contain 448 amino acids, to have a predicted molecular weight of 51.4 kDa and to contain six potential N-glycosylation sites (Ogi *et al*, 1993). The amino acid sequence showed 92.5 % homology with the rat PAC1 receptor and 55.3 % homology with the human VPAC receptor (Ogi *et al*, 1993). Ligand binding studies performed on transfected cell-lines demonstrated 1000 times higher affinity of the receptor for PACAP than for VIP (Ogi *et al*, 1993). VIP was also at least 1000 times less potent than PACAP in stimulating cAMP production (Miyata *et al*, 1989; Ogi *et al*, 1993).

Splice variants of the human PAC1 receptor have also been discovered, which are closely associated with the four main rat PAC1 receptor splice variants (Pisegna & Wank, 1996). These four variants, null, SV-1, SV-2 and SV-3, are formed from alternative splicing between two exons in the third intracellular loop region, leading to the different insertions of up to two 28 amino acid cassettes, as in the rat (Spengler *et al*, 1993; Pisegna & Wank, 1996). Thus, the null, SV-1, SV-2 and SV-3 forms in the human correspond to the *short*, *hip*, *hop* and *hiphop* forms in the rat. However, differences exist between the human and rat splice variants in that all of the human variants bind PACAP27 and PACAP38 with similar affinity and potency for stimulating adenylate cyclase, unlike the rat variants (Pisegna & Wank, 1996). However, each human splice variant differed in the efficacy (maximal stimulation) for PLC activation and total inositol phosphate formation, with the order of efficacy being SV-2 > null > SV-1 > SV-3 (Pisegna & Wank, 1996).

Expression of the PAC1 receptor mRNA, determined by Northern blot analysis, was abundant in the brain, with little in peripheral tissues (Hashimoto *et al*, 1993; Ogi *et al*, 1993) except a relatively weak level of expression in the adrenal

gland (Hashimoto *et al*, 1993). A high affinity binding site with the characteristics of a PAC1 receptor has also been identified on adrenal medullary chromaffin cells by receptor autoradiography (Shivers *et al*, 1991).

The aims of this chapter were to determine whether VIP-stimulated cortisol and cAMP production from H295R cells required activation of a VIP-specific receptor or occurred by one of the other mechanisms discussed above.

The rationale for β -adrenoceptor involvement was based on reports that VIP induced catecholamine release from chromaffin tissue, with subsequent activation of β -adrenoceptors on adrenocortical cells, leading to increased steroidogenesis (section 4.1.1). This mechanism was considered unlikely to account for VIP-stimulated cortisol release from H295R cells because the cell-line is not known to be contaminated with chromaffin cells. Nevertheless, it was considered important to exclude a β -adrenoceptor-mediated mechanism from this transformed cell-line, since there are no published reports that H295R cell cultures do not contain chromaffin cells or that they do not contain β -adrenoceptors.

A mechanism involving direct activation of a PAC1 receptor was investigated because VIP and PACAP share 68% homology in their amino acid sequences (Miyata *et al*, 1989) and VIP and PACAP receptors belong to the same receptor family (see 6.1.1). PACAP is capable of activating VPAC receptors with similar affinity and potency to VIP itself (*Table 6.1*). In addition, VIP can activate the PAC1 receptor but with 100-1000-fold less potency than PACAP. If VIP increases cortisol production from H295R cells via a PAC1 receptor-mediated mechanism, it would therefore be predicted that PACAP would stimulate cortisol secretion and cAMP accumulation with greater potency than VIP.

Experiments in this chapter were performed using H295R cells which had been pretreated with forskolin (10 μ M, 72 h) in keeping with the conditions used for the cortisol and second messenger studies earlier (see chapter 5.0).

The effects of the β -adrenoceptor antagonist, propranolol, on 4 h VIP-stimulated cortisol secretion and cAMP production was examined, along with the

direct effects of the β -adrenoceptor agonists, adrenaline and isoprenaline, on cortisol and cAMP responses. A concentration of 3.3×10^{-8} M VIP was used, unless otherwise stated, as this concentration resulted in maximal cortisol secretion and maximal cAMP production from H295R cells in previous experiments (chapter 5.0).

A comparison was made between the concentration-responses curves for cortisol and cAMP following treatment of cells with either VIP or PACAP38, to determine whether the PAC1 receptor is involved in these responses from H295R cells.

The effects of the VPAC1 receptor antagonists, C1-Phe⁶, Leu¹⁷-VIP and acetyl-tyr-GRF-amide, on VIP-mediated cortisol and cAMP responses were also investigated along with an examination of the direct effects of the VPAC2 receptor superagonist, RO-25-1553, on cortisol and cAMP production by H295R cells.

Finally, the gene expression for the VPAC1 receptor, VPAC2 receptor and PAC1 receptor in H295R cells was studied using RT-PCR.

6.2 Results

6.2.1 Effect of propranolol on VIP-stimulated cortisol and cAMP production

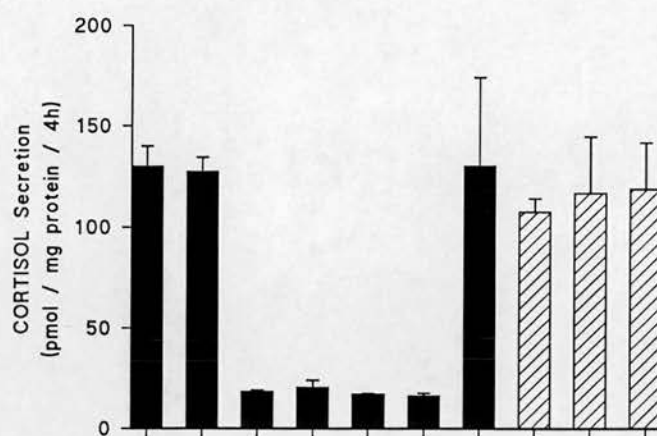
In order to exclude the possibility that the effects of VIP on cortisol and cAMP production by H295R cells were mediated by β -adrenoceptors, cells were treated with VIP (3.3×10^{-8} M) in the absence or presence of various concentrations (10^{-8} M - 10^{-6} M) of the non-selective β -adrenoceptor antagonist, propranolol. Propranolol did not alter VIP-stimulated cortisol or cAMP production at any concentration used ($P \geq 0.05$) (*Figure 6.1*). The effects of propranolol on basal and forskolin-stimulated cortisol and cAMP production were also determined to rule out the possibility of a non-specific or toxic effect of propranolol on cortisol or cAMP formation. Propranolol did not affect basal or forskolin-stimulated cortisol or cAMP production ($P \geq 0.05$).

6.2.2 Effect of the β -adrenoceptor agonists, adrenaline and isoprenaline, on cortisol and cAMP production by H295R cells

The direct effects of the non-selective and physiological β -adrenoceptor agonist, adrenaline, on cortisol and cAMP production by H295R cells was investigated. Adrenaline failed to stimulate cortisol secretion or cAMP production over basal levels at any of the concentrations tested (10^{-8} M - 10^{-6} M) ($P \geq 0.05$) in contrast to forskolin (10^{-5} M) treatment and VIP (3.3×10^{-8} M) treatment ($P < 0.01$) (*Figure 6.2*).

Cells were also treated with the synthetic, non-selective, β -adrenoceptor agonist isoprenaline (10^{-8} M - 10^{-5} M), which has a higher potency than adrenaline for β -adrenoceptor activation. Isoprenaline also failed to increase cortisol secretion or cAMP production over basal from H295R cells ($P \geq 0.05$) (*Figure 6.3*).

(A)



(B)

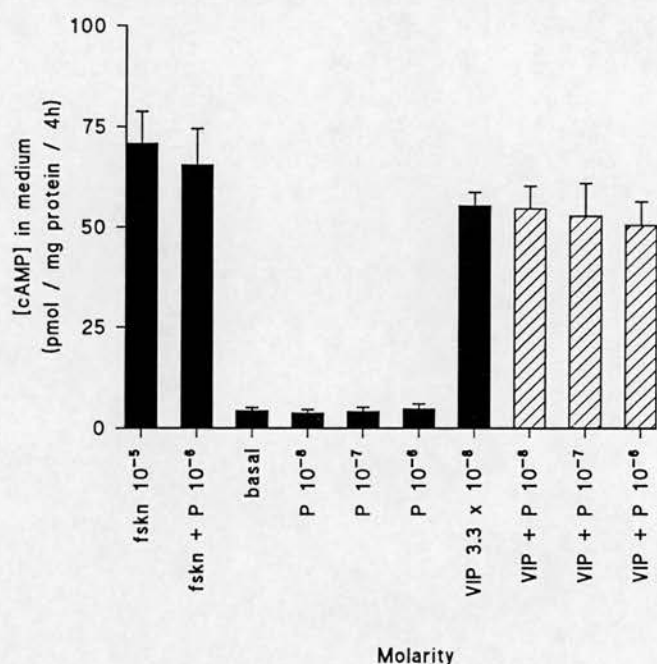
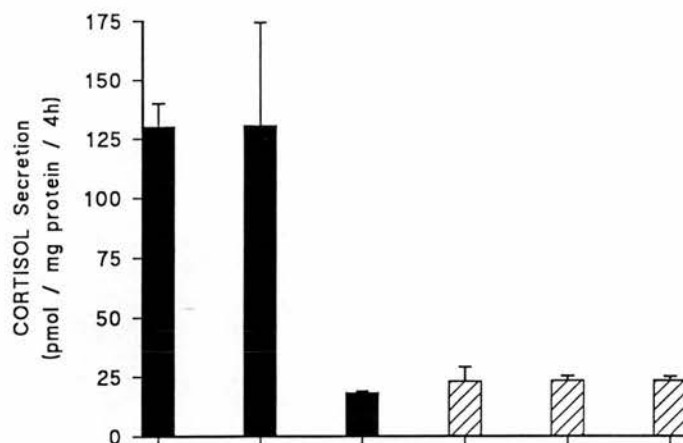


Figure 6.1

Effect of the non-selective β -adrenoceptor antagonist, propranolol, on (A) VIP-stimulated cortisol secretion and (B) VIP-stimulated cAMP production from H295R cells. Forskolin-pretreated cells were washed twice and treated with either VIP ('VIP', 3.3×10^{-8} M), forskolin ('fskn', 10^{-5} M) or serum-free growth medium alone ('basal') with or without propranolol ('P', 10^{-8} M, 10^{-7} M or 10^{-6} M) for 4 h. Each treatment was performed in triplicate within each individual experiment. Medium was assayed for cortisol and cAMP content by RIA (Methods, chapter 2.0). Cortisol and cAMP data were corrected with respect to total cellular protein. Results shown are mean (\pm s.d.) of triplicate measurements from a representative experiment from $n=3$ separate experiments.

(A)



(B)

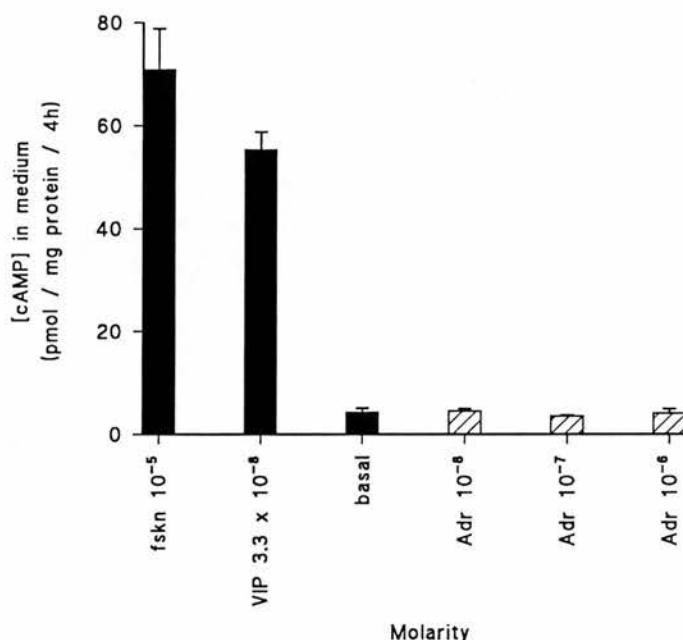
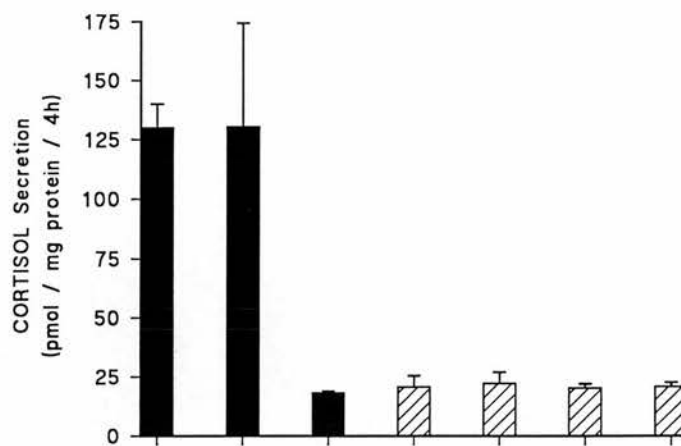


Figure 6.2

Effect of adrenaline on (A) cortisol secretion and (B) cAMP production from H295R cells compared with basal, VIP-stimulated and forskolin-stimulated cortisol secretion. Forskolin-pretreated cells were washed twice and treated with either adrenaline ('Adr', hatched bars, 10^{-8} M, 10^{-7} M or 10^{-6} M), VIP ('VIP', 3.3×10^{-8} M), forskolin ('fskn', 10^{-5} M) or serum-free growth medium alone ('basal') for 4 h. Each treatment was performed in triplicate within each individual experiment. Medium was assayed for cortisol and cAMP content by RIA (Methods, chapter 2.0). Cortisol and cAMP data were corrected with respect to total cellular protein. Results shown are mean (\pm s.d.) of triplicate measurements from a representative experiment from $n=3$ separate experiments.

(A)



(B)

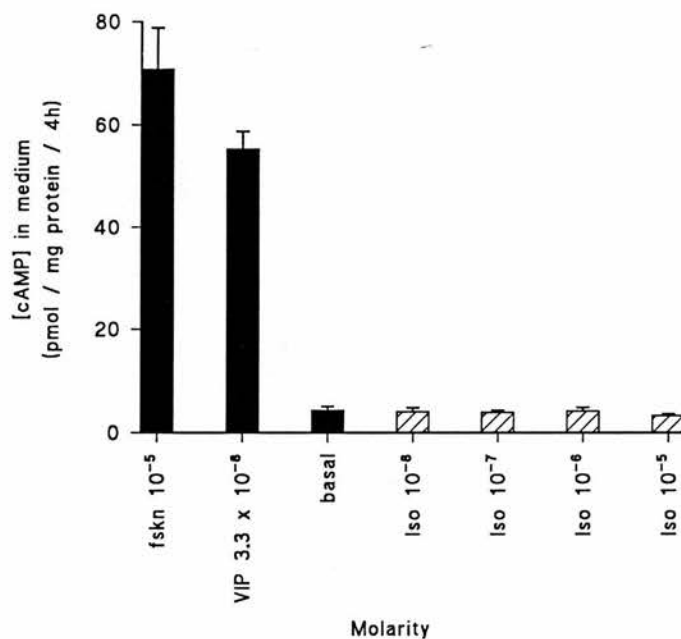


Figure 6.3

Effect of isoprenaline on (A) cortisol secretion and (B) cAMP production from H295R cells.

Forskolin-pretreated cells were washed twice and treated with either isoprenaline ('Iso', hatched bars, 10^{-8} - 10^{-5} M), VIP ('VIP', 3.3×10^{-8} M), forskolin ('fskn', 10^{-5} M) or serum-free growth medium alone ('basal') for 4 h. Each treatment was performed in triplicate within each individual experiment. Medium was assayed for cortisol and cAMP content by RIA. Data were corrected with respect to total cellular protein. Results shown are mean (\pm s.d.) of triplicate measurements from one experiment. Similar results were obtained from a second experiment.

Adrenaline (10^{-8} M - 10^{-6} M) also failed to stimulate cortisol or cAMP production over basal levels from H295R cells which had not been pretreated with forskolin ($P \geq 0.05$) (*Figure 6.4*).

6.2.3 Effect of PACAP38 on cortisol and cAMP production from H295R cells

The cortisol and cAMP concentration-response curves from H295R cells following treatment with either PACAP38 (10^{-11} M - 10^{-7} M) or VIP (10^{-11} M - 10^{-7} M) were compared. PACAP38 and VIP both increased cortisol secretion and cAMP production in a concentration-dependent manner (see *Figures 6.5* and *6.6* for cortisol and cAMP concentration-response curves respectively).

The cortisol concentration-response curves were similar (*Figure 6.5*). The threshold concentrations for cortisol secretion were 10^{-11} M for both PACAP38 and VIP, maximal secretion occurring at a concentration of 3.3×10^{-9} M for PACAP38 and 3.3×10^{-8} M for VIP. The pEC50 values (negative logarithm to the base 10 of the concentration (Molar) producing a half-maximal response) for PACAP38 and VIP were 9.8 ± 0.06 and 9.4 ± 0.43 respectively ($n=3$ for both) (*Table 6.2*).

The cAMP concentration-response curves for PACAP38 and VIP were also similar (*Figure 6.6*). Maximal cAMP production occurred at a concentration of 10^{-8} M for PACAP38 and 3.3×10^{-8} M for VIP. Threshold stimulation for cAMP production occurred at 10^{-11} M for both PACAP38 and VIP and the pEC50 value for VIP-induced cAMP production was 8.7 ± 0.46 M ($n=3$ individual experiments), compared with a pEC50 value of 9.3 ± 0.05 M ($n=3$ individual experiments) for PACAP38-stimulated cAMP production (*Table 6.2*).

6.2.4 Effect of VPAC1 receptor antagonists on VIP-stimulated cortisol and cAMP production

Figure 6.7 shows the effect of the VPAC1 receptor antagonist, Cl-Phe⁶, Leu¹⁷-VIP (CPLV) ($0.05 \mu\text{M}$ - $5 \mu\text{M}$) on VIP-stimulated cortisol and cAMP production. Cells were treated with the antagonist for 30 mins prior to the addition

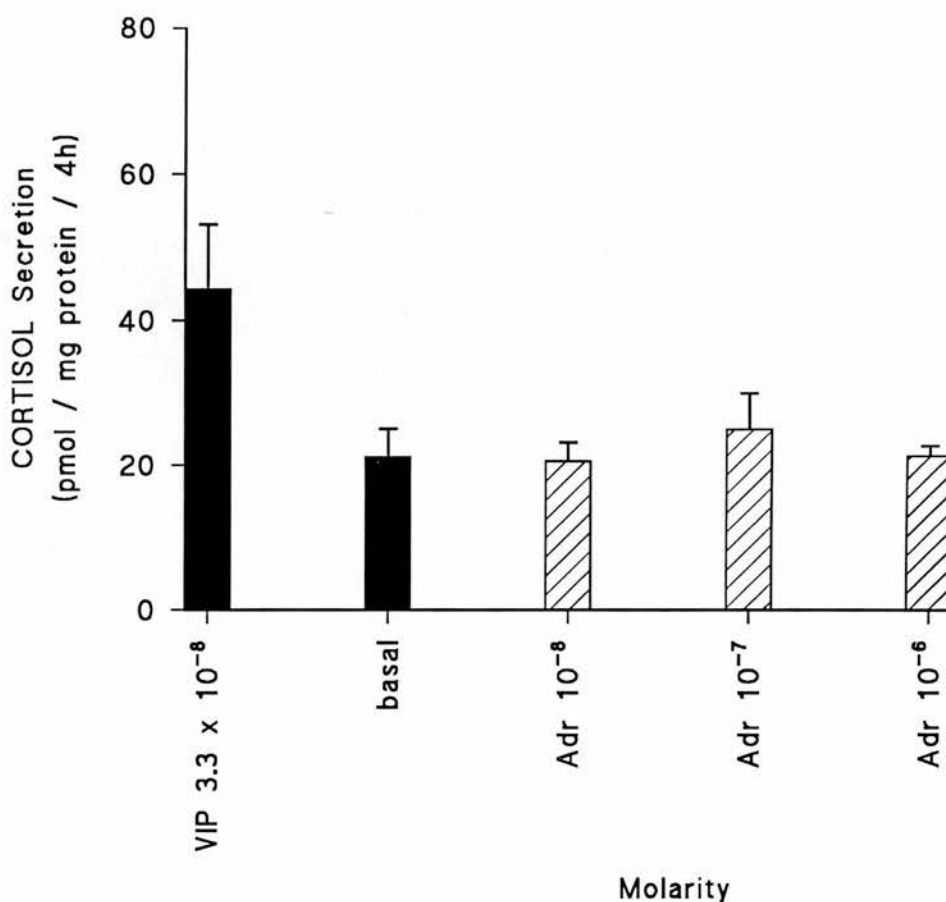


Figure 6.4

Effect of adrenaline on cortisol secretion from non-forskolin-pretreated H295R cells (cells not pretreated with forskolin (10^{-5} M) for 72 h). The protocol was the same as for forskolin-pretreated cells except that no forskolin was added to the pretreatment medium. After 72 h cells were washed twice and treated with either adrenaline ('Adr', hatched bars, 10^{-8} M, 10^{-7} M or 10^{-6} M), VIP ('VIP', 3.3×10^{-8} M) or serum-free growth medium alone ('basal') for 4 h. Each treatment was performed in triplicate. Medium was assayed for cortisol content by RIA. Cortisol data were corrected with respect to total cellular protein. Results shown are mean (\pm s.d.) of triplicate measurements from one experiment.

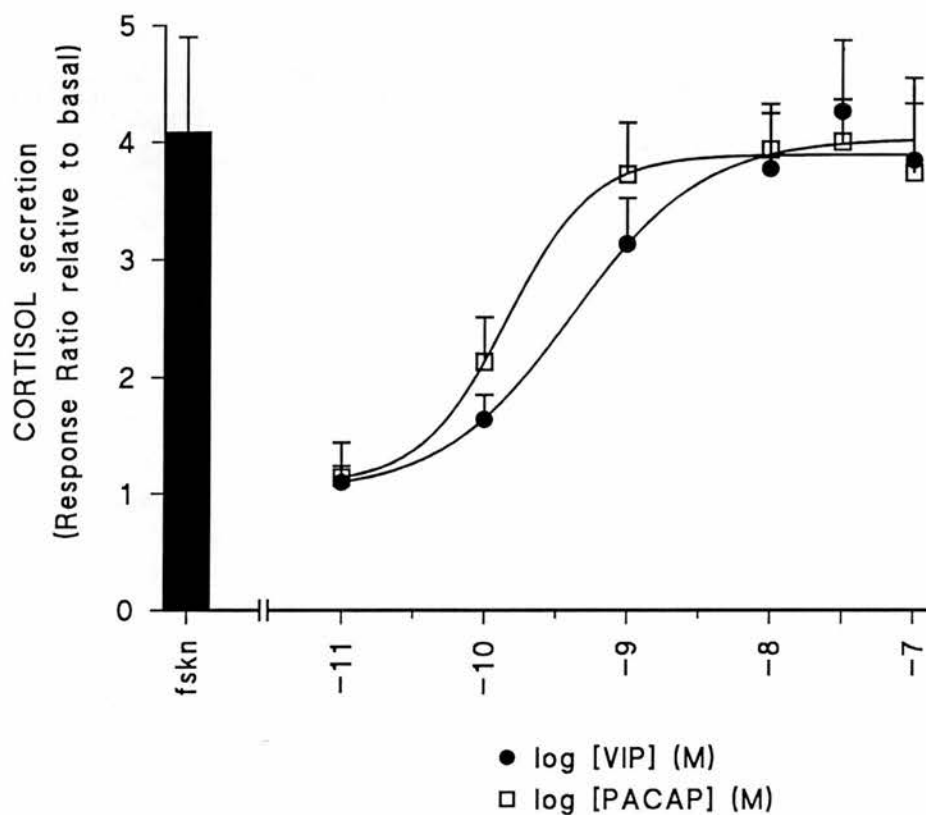


Figure 6.5

Comparison of concentration-dependent increases in cortisol secretion from H295R cells following 4 h treatment with VIP, PACAP38 or forskolin. Forskolin-pretreated cells were washed thoroughly, then treated with VIP (10^{-11} - 10^{-7} M, ●), PACAP38 (10^{-11} - 10^{-7} M, □) or forskolin (10^{-5} M, bar labelled fskn) for 4 h. Treatments were performed in triplicate within each experiment. Cortisol content of medium was assayed by RIA and corrected with respect to total cellular protein. Combined data from n=3 experiments is expressed as mean (\pm s.d.) Response Ratios (response to stimulus / basal secretion).

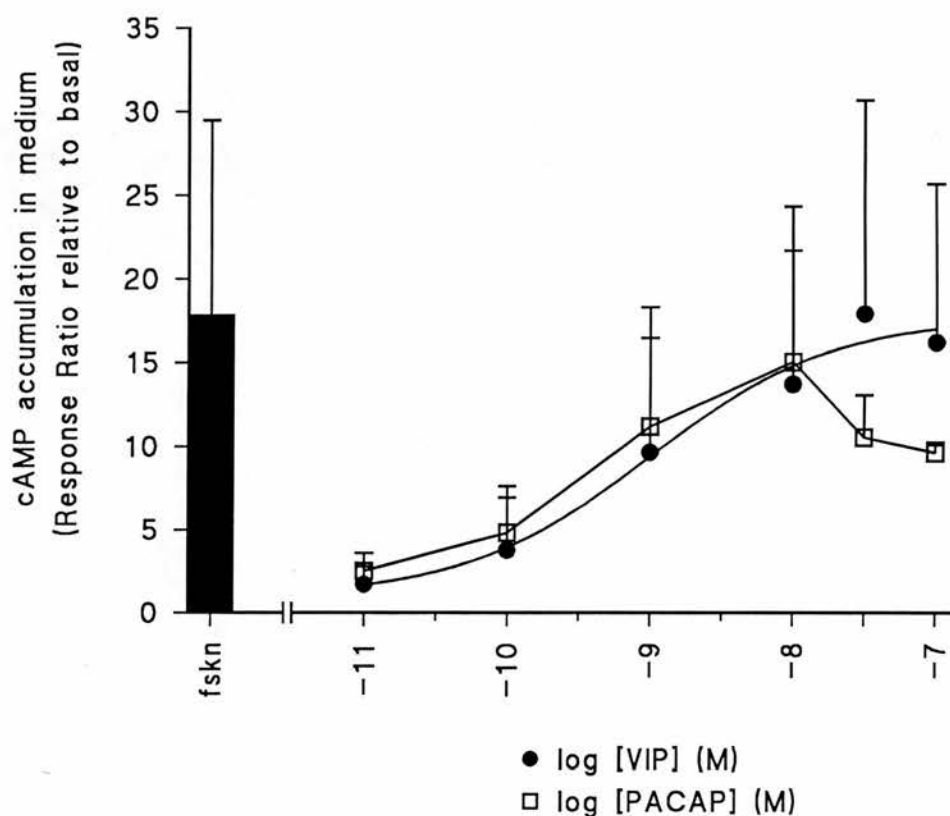


Figure 6.6

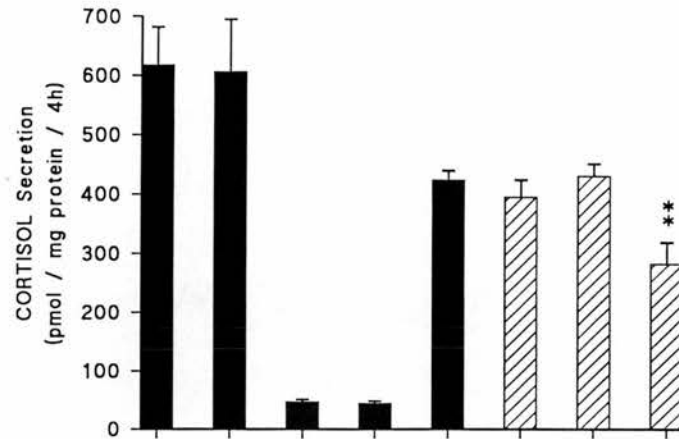
Comparison of concentration-dependent increases in cAMP production from H295R cells following 4 h treatment with VIP, PACAP38 or forskolin. Forskolin-pretreated cells were washed thoroughly, then treated with VIP (10^{-11} - 10^{-7} M, ●), PACAP38 (10^{-11} - 10^{-7} M, □) or forskolin (10^{-5} M, bar labelled fskn) for 4 h. Treatments were performed in triplicate within each experiment. cAMP content of acidified medium was assayed by RIA and corrected with respect to total cellular protein. Combined data from n=3 experiments is expressed as mean (\pm s.d.) Response Ratios (response to stimulus / basal secretion).

pEC50 (+/- s.d.) for VIP-induced cortisol and cAMP production		
	CORTISOL response	cAMP response
<i>Experiment 1</i>	9.533 (0.10)	9.193 (0.1)
<i>Experiment 2</i>	8.921 (0.12)	8.294 (0.33)
<i>Experiment 3</i>	9.640 (0.19)	8.591 (0.21)
MEAN pEC50 (+/- s.d.)	9.365 (0.43)	8.693 (0.46)
pEC50 (+/- s.d.) for PACAP-induced cortisol and cAMP production		
	CORTISOL response	cAMP response
<i>Experiment 1</i>	9.863 (0.09)	9.337 (0.03)
<i>Experiment 2</i>	9.786 (0.07)	9.266 (0.08)
<i>Experiment 3</i>	9.746 (0.02)	9.361 (0.03)
MEAN pEC50 (+/- s.d.)	9.798 (0.06)	9.321 (0.05)

Table 6.2

Comparison of pEC50 values for cortisol secretion and cAMP accumulation following treatment of H295R cells with either VIP or PACAP38. Forskolin-pretreated cells were washed and treated with VIP (10^{-11} M - 10^{-7} M) or PACAP38 (10^{-11} M - 10^{-7} M) for 4 h. Medium was collected and assayed for cortisol and cAMP content as described previously (see Methods, section 2.2). Concentration-response curves were plotted and the pEC50 (negative logarithm to the base 10 of the concentration (Molar) producing a half-maximal response) calculated for each experiment from n=3 experiments.

(A)



(B)

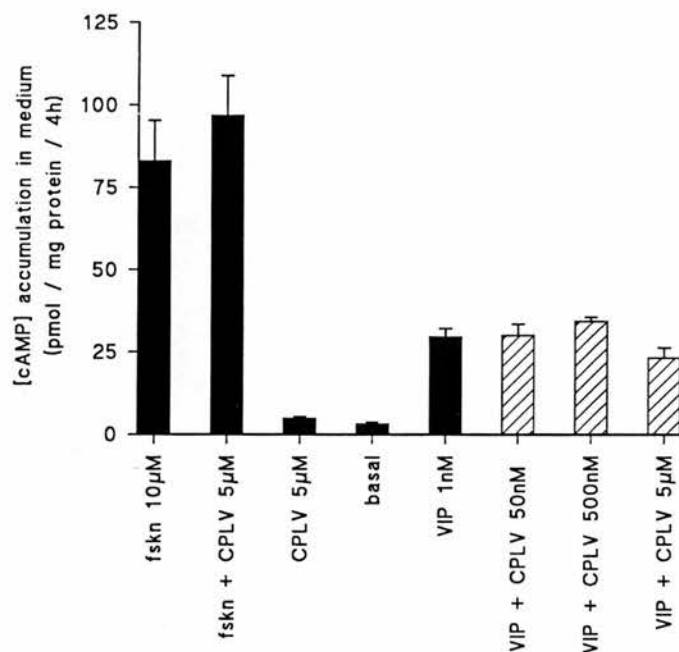


Figure 6.7

Effects of VPAC1 receptor antagonist, *Cl-Phe*⁶, *Leu*¹⁷-VIP (CPLV) on VIP-stimulated production of (A) cortisol and (B) cAMP. Cells were pre-incubated with CPLV (0.05 - 5 µM) for 30 mins in serum-free growth medium prior to the addition of VIP (1 nM) for a further 4 h. Cells were also treated with VIP alone (1 nM), forskolin (10 µM, 'fskn'), CPLV (5 µM) alone and fskn plus CPLV (5 µM) together. Data presented are mean (± s.d.) of triplicate measurements from one experiment. A statistically significant response compared with VIP-stimulated response is indicated by **P<0.02. Similar data was obtained in a second experiment except that 5 µM CPLV did not significantly decrease VIP-stimulated cortisol secretion (P≥0.05).

of VIP to allow the antagonist to bind to the receptors before VIP. A sub-maximal concentration of VIP (1 nM) was used to compete with the antagonist for VPAC1 receptors. CPLV at concentrations of 0.05 μ M or 0.5 μ M failed to attenuate the cortisol and cAMP responses to VIP ($P \geq 0.05$). A small decrease (34 %) in the cortisol response to VIP was seen in the presence of 5 μ M CPLV ($P < 0.02$), but this effect was only observed in 1/2 experiments. A similar experiment was performed using a different VPAC1 receptor antagonist, acetyl-tyr-GRF-amide (A-T-GRF-A) (0.05 μ M - 5 μ M). This antagonist failed to decrease cortisol or cAMP responses to VIP (1 nM) at any concentration tested ($P \geq 0.05$) (*Figure 6.8*). Neither CPLV (5 μ M) nor A-T-GRF-A (5 μ M) altered basal levels of cortisol secretion or cAMP production or 10 μ M forskolin-stimulated levels of cortisol secretion or cAMP production.

6.2.5 Effects of VPAC2 superagonist, RO 25-1553, on cortisol and cAMP production by H295R cells

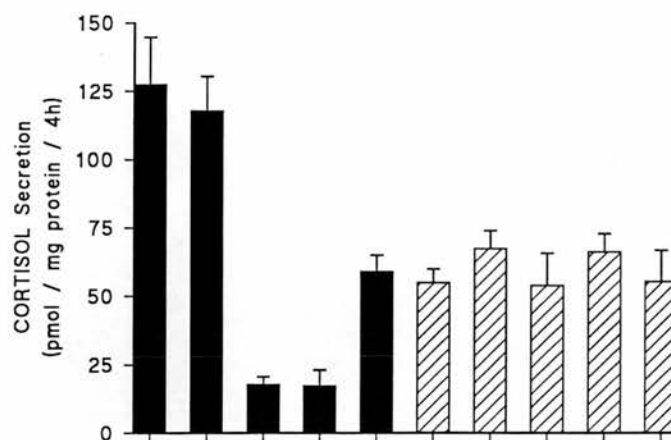
Figure 6.9 shows the effects of the VPAC2 receptor superagonist, RO-25-1553, on cortisol secretion and cAMP production by H295R cells. RO-25-1553 (10^{-9} M, 10^{-8} M) failed to stimulate either cortisol or cAMP production over basal levels ($P \geq 0.05$). However, 10^{-7} M RO-25-1553 increased cortisol secretion and cAMP accumulation in two out of three experiments ($P < 0.01$ in each experiment for both cortisol and cAMP).

6.2.6 Analysis of VPAC1 receptor, VPAC2 receptor and PAC1 receptor gene expression in H295R cells by RT-PCR

Figure 6.10 shows the products of RT-PCR analysis, visualised on an ethidium bromide-stained 0.8 % agarose gel, of total RNA extracted from H295R cells. The cells were treated with or without forskolin (10 μ M) for 72 h prior to extraction of the RNA.

A band of the expected molecular weight for the VPAC1 receptor (440 bp) was not obtained from the VPAC1 receptor positive control material (cDNA obtained from a human lung cDNA library) with the primers used (lane 1 of *Figure 6.10(a)*),

(A)



(B)

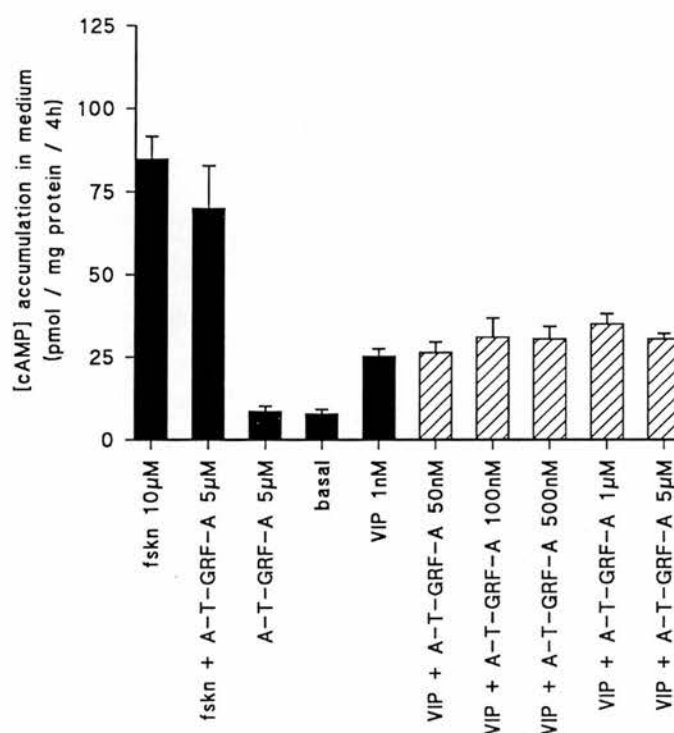
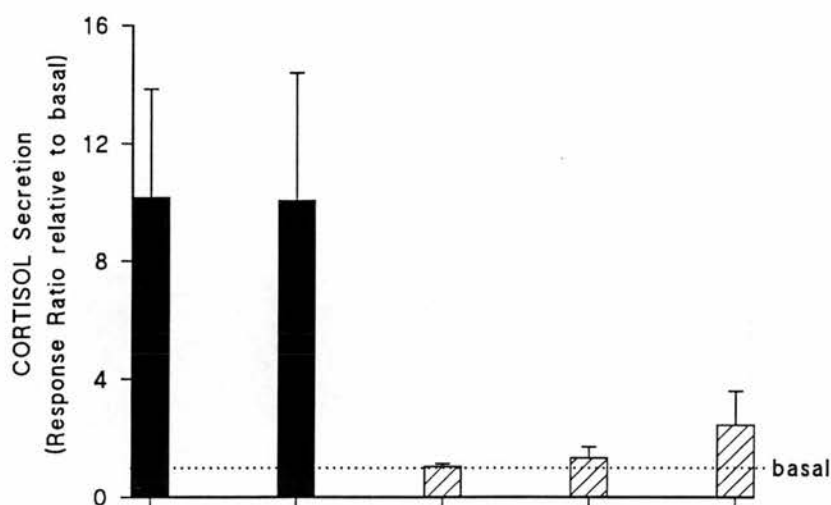


Figure 6.8

Effects of VPAC1 receptor antagonist, Acetyl-Tyr-GRF-amide (A-T-GRF-A) on VIP-stimulated production of (A) cortisol and (B) cAMP. Forskolin-pretreated cells were washed twice then incubated with A-T-GRF-A (0.05 - 5 µM) for 30 mins in serum-free growth medium prior to the addition of VIP (1 nM) for a further 4 h. Cells were also treated with VIP alone (1 nM), forskolin (10 µM, 'fskn'), A-T-GRF-A (5 µM) alone and fskn plus A-T-GRF-A (5 µM). Data presented are means (\pm s.d.) of triplicate measurements from one experiment.

(A)



(B)

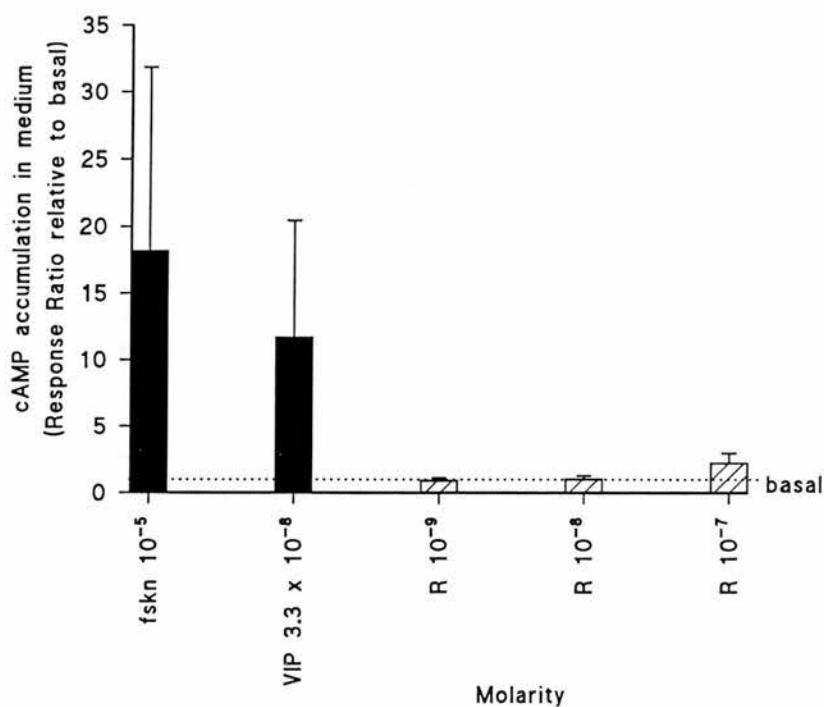


Figure 6.9

Effects of VPAC2 receptor superagonist, RO-25-1553, on production of (A) cortisol and (B) cAMP by H295R cells. Forskolin-pretreated cells were washed twice and then treated with RO-25-1553 (10^{-9} , 10^{-8} or 10^{-7} M, 'R'), VIP (3.3×10^{-8} M) or forskolin (10^{-5} M, 'fskn') in serum-free growth medium for 4 h. Cortisol and cAMP contents of medium were assayed by RIAs and corrected with respect to total cellular protein. Combined data from $n=3$ experiments, triplicate incubations performed in each, are expressed as mean (\pm s.d.) Response Ratios (response to stimulus / basal secretion).

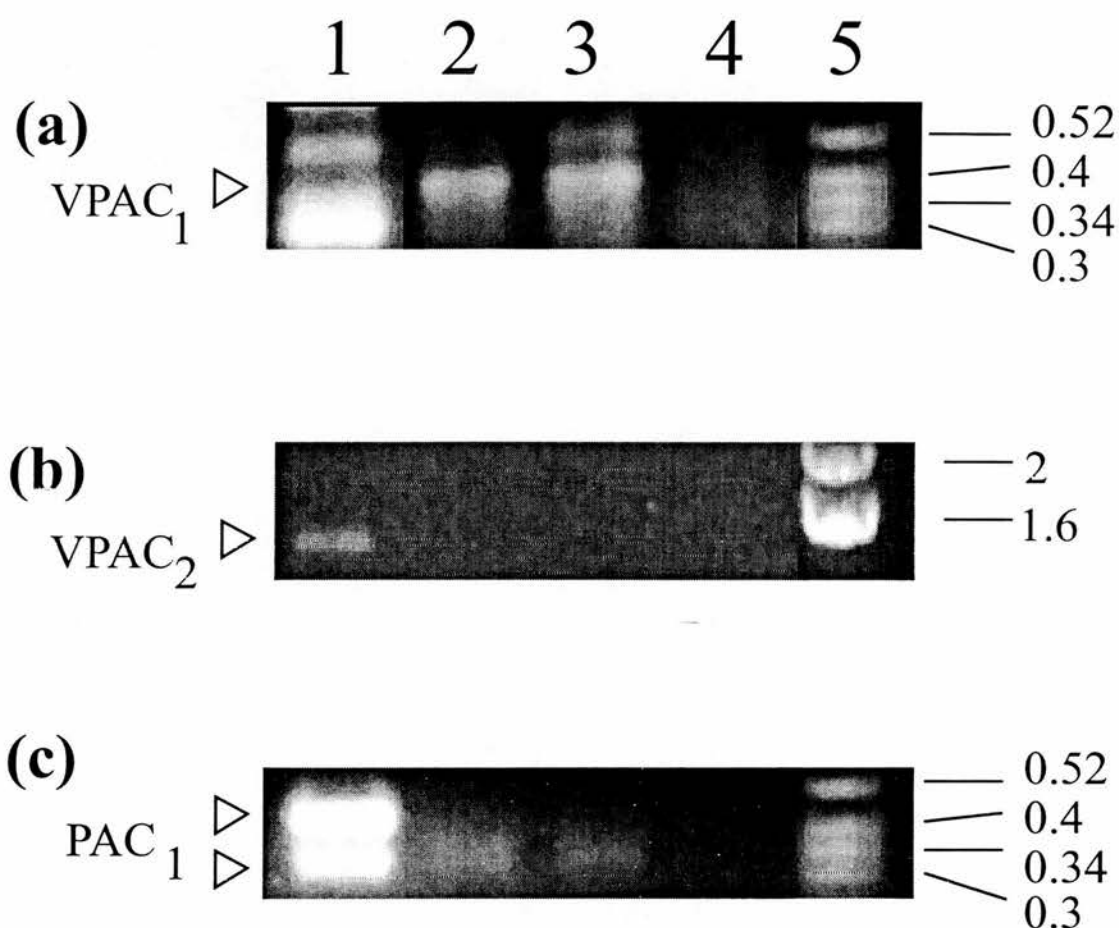


Figure 6.10

RT-PCR detection of (a) VPAC₁, (b) VPAC₂ and (c) PAC₁ mRNA in H295R cells. Lane 1 corresponds to PCR of positive control material ((a) human lung cDNA library, (b) human placental cDNA library and (c) human brain cDNA library), lane 2 corresponds to RT-PCR of control H295R cells, lane 3 corresponds to RT-PCR of cells treated with forskolin (10 μ M) for 72 h, lane 4 corresponds to PCR of negative control material (no DNA) and lane 5 corresponds to 1 kb DNA molecular weight markers.

although bands of the expected weight were obtained following RT-PCR of total RNA from H295R cells treated without and with forskolin (see lanes 2 and 3, respectively, of *Figure 6.10(a)*).

PCR of cDNA from a human placenta cDNA library (VPAC2 receptor positive control material) yielded a product of the expected size for the VPAC2 receptor (1563 bp) (lane 1 of *Figure 6.10(b)*). No equivalent bands were observed from RT-PCR of H295R total RNA (lanes 2 and 3 of *Figure 6.10(b)*).

PCR of PAC1 positive control material (cDNA from human brain cDNA library) gave rise to two bands of 335bp and 440bp as expected (lane 1 of *Figure 6.10(c)*). Corresponding, but relatively very faint, bands were observed from RT-PCR of total RNA from untreated (control) H295R cells and forskolin-treated H295R cells (lanes 2 and 3, respectively, of *Figure 6.10(c)*).

The negative control (no DNA) lanes were all blank as expected (lane 4 of *Figure 6.10(a), (b) and (c)*).

6.3 Discussion

Several studies have suggested that VIP exerts an indirect action on the adrenal cortex via catecholamine release from medullary chromaffin cells and subsequent activation of β -adrenoceptors on adrenocortical cells, leading to an increase in steroid secretion (section 4.1.1). Therefore, it was considered important to determine whether the effects of VIP on cortisol and cAMP observed in H295R cells required activation of β -adrenoceptors, either directly by VIP or as a result of VIP-stimulated catecholamine release from the transformed adrenocortical cells or from cells of chromaffin lineage. The non-selective β -adrenoceptor antagonist, propranolol, did not alter the cortisol or cAMP responses to VIP, consistent with these VIP-mediated effects not requiring the activation of β -adrenoceptors. Indeed, both the physiological catecholamine, adrenaline, and the non-selective β -adrenoceptor agonist, isoprenaline, failed to stimulate either cAMP production or cortisol secretion from H295R cells. Since Bornstein et al (1996) have reported an apparent β -adrenoceptor-mediated effect on steroid secretion from cultured normal human adrenocortical cells, this suggests a loss of functional β -adrenoceptors from this cell-line. To rule out the possibility that the lack of response to adrenaline was due to cAMP-mediated β -adrenoceptor downregulation during the 72 h pretreatment phase with forskolin, non-pretreated cells were also treated with adrenaline and VIP. VIP caused an increase in cortisol secretion which was diminished but still significant ($P < 0.01$) compared with forskolin-pretreated cells (consistent with previous findings in non-pretreated cells). No cortisol response to adrenaline was observed. This supports the suggestion that the H295R cell-line lacks functional β -adrenoceptors.

Comparison of cortisol and cAMP responses following VIP treatment and PACAP treatment demonstrated similar potencies of the two peptides for both responses. This observation is consistent with VIP effects being mediated by activation of a VIP-specific receptor (VPAC receptor) rather than a PAC1 receptor.

Further studies using VPAC1 receptor antagonists and a VPAC2 receptor superagonist failed to clearly establish which VIP receptor subtype(s) was implicated in the steroid and cAMP responses from H295R cells. At the time the experiments

were performed a VPAC2 receptor antagonist was not available. The VPAC2 receptor superagonist, RO 25-1553, did not alter cortisol secretion or cAMP accumulation over basal from H295R cells at concentrations of 10^{-9} M or 10^{-8} M. RO 25-1553 has been shown to be 3-fold more potent than VIP on the human VPAC2 receptor, 600-fold less potent than VIP on the human VPAC1 receptor and 10-fold less potent than VIP on the PAC1 receptor (Gourlet *et al*, 1997c). Whilst a concentration of 10^{-7} M RO 25-1553 did increase cortisol and cAMP levels over basal in two out of three experiments performed, this effect was much smaller than that seen in response to VIP and was probably related to the partial agonist action of RO 25-1553 on human VPAC1 receptors at high concentrations (Gourlet *et al*, 1997c). These results suggest that VIP-induced steroid and cAMP responses were not mediated through activation of VPAC2 receptors. The presence of VPAC2 receptors on this cell-line cannot be excluded though data using RO 25-1553 and from RT-PCR experiments would argue that expression of this receptor subtype in H295R cells, if any, must be low.

The VPAC1 receptor antagonists used failed to inhibit steroid secretion or cAMP accumulation from H295R cells. At the time these experiments were performed, little data was available on the affinity and potency of these antagonists for human VPAC receptors. However, both of these antagonists exhibited a low affinity for VIP receptor binding in rat mesenteric artery, anterior pituitary, brain synaptosomes, liver and bovine coronary artery tissues (Rorstad *et al*, 1990) and micromolar concentrations of CPLV were required to antagonise VIP-stimulated amylase release from guinea pig pancreatic preparations (Pandol *et al*, 1986). Since the experiments in this chapter were performed, a highly selective and potent human and rat VPAC1 receptor antagonist, PG 97-269, has been developed with an IC_{50} value in the nM range (Gourlet *et al*, 1997a). Use of this antagonist would help determine the presence of VPAC1 receptors on the H295R cell-line. In addition, a high affinity selective human VPAC1 receptor agonist, $[K^{15}, R^{16}, L^{27}]VIP(1-7)/GRF(8-27)$ has now been described (Gourlet *et al*, 1997b).

A species-specific difference in VIP receptor subtypes expressed in the adrenal may exist. Usdin *et al* (1994) demonstrated a predominance of VPAC1

receptor mRNA in the rat adrenal medulla with a higher proportion of VPAC2 receptor mRNA in the rat adrenal cortex. However, Adamou *et al* (1995) noted that the VPAC2 receptor transcript was highly expressed in human skeletal muscle, in contrast to rat skeletal muscle where VPAC2 receptor mRNA was not detected (Usdin *et al*, 1994).

In an attempt to extend the studies using VPAC1 receptor and VPAC2 receptor antagonists and to further clarify which receptor(s) are present on the H295R cell-line, RT-PCR experiments were performed to detect the presence of VPAC1 receptor, VPAC2 receptor and PAC1 receptor mRNA transcripts. The suggestion that the steroid and cAMP responses were mediated mainly through a VPAC receptor rather than a PAC1 receptor was supported by the RT-PCR data obtained. PAC1 receptor mRNA was barely detectable in H295R cells, indicating a very low level of expression of this receptor in these cells, if any. The presence of two bands in the positive control material corresponds to splice variants of the PAC1 receptor, probably the null form and the SV-1 or SV-2 form.

RT-PCR data indicated that H295R cells expressed transcripts for the VPAC1 receptor but little, if any, VPAC2 receptor mRNA. Unfortunately, the RT-PCR data obtained in this study was not conclusive. Sequencing of the bands from the PCR reaction would confirm the identity of the PCR product as VPAC1 receptor mRNA.

However, the presence of VPAC1 receptors on this cell-line is supported by studies involving RT-PCR of H295R cell mRNA using degenerate primers for the VIP / PACAP / PTH receptor family, followed by sequencing of a selection of the cloned products, which demonstrated a predominance of the VPAC1 receptor transcript. The VPAC2 receptor transcript was not detected in any of the 9 clones tested and the PAC1 receptor transcript was detected in 1/9 Clones (Dr. Eve Lutz, *personal communication*).

The results from this chapter lend support for a direct action of VIP on cortisol secretion from H295R cells by a direct receptor-mediated action, probably a VPAC receptor rather than a PAC1 receptor and separate from any β -adrenoceptor-mediated effect. In the light of the discovery that VIP and PACAP share some receptors, it was necessary to determine whether the actions attributed to VIP so far

were mediated by VPAC receptors or PAC1 receptors since the presence of PAC1 receptors in a tissue may indicate that PACAP is the more biologically important of the two peptides for some functions. For example, radioligand binding studies and autoradiography have indicated that the predominant VIP / PACAP-binding site in the rat adrenal medulla is a PAC1 receptor (Shivers *et al*, 1991; Moller & Sundler, 1996), found on chromaffin cells. PAC1 receptor mRNA has also been detected in the adrenal gland (Hashimoto *et al*, 1993), in medullary chromaffin cells but not in the adrenal cortex (Moller & Sundler, 1996). Furthermore, PACAP has been shown to stimulate catecholamine release from the adrenal medulla of rats (Watanabe *et al*, 1992), dogs (Gaspo *et al*, 1997), cultured bovine chromaffin cells (Babinski *et al*, 1996) and the PC12 rat chromaffin cell-line (Taupenot *et al*, 1998) and these effects appeared to be mediated by a PAC1 receptor. Whilst VPAC receptor mRNA has been detected in the adrenal medulla of rats (Usdin *et al*, 1994), the proportion of VPAC receptors was much lower than PAC1 receptors (Shivers *et al*, 1991). Interestingly, PACAP-immunoreactive nerve fibres have been reported in the medulla but no PACAP immunoreactivity was reported in chromaffin cells or ganglion cells. In contrast, VIP-immunoreactivity was observed in chromaffin and ganglionic tissues in rat adrenals whereas VIP-immunopositive nerve fibres were concentrated in the cortex (Moller & Sundler, 1996). Thus, it is possible that PACAP is more biologically important in the adrenal medulla than VIP, in terms of stimulating catecholamine secretion. Hinson *et al* (1992) obtained catecholamine and steroid responses from rat zg-capsule preparations using a high concentration (10 μ M) of VIP. Although the high concentration of VIP required to stimulate steroidogenesis may be attributed to the scarcity of chromaffin tissue in the preparation, it could also be due to an action of VIP on a PAC1 receptor rather than a VPAC receptor. Edwards & Jones (1993a) noted that, whilst VIP infusion into functionally hypophysectomised calves resulted in a cortisol response equivalent to that elicited by ACh, VIP was not similarly potent in stimulating catecholamine secretion. Thus, it may be that an indirect action of VIP on steroid secretion requires activation of PAC1 receptors on chromaffin cells and that a direct action depends upon activation of VPAC receptors on adrenocortical cells. The presence of both

PACAP and VIP in the adrenal medulla may allow for several levels of control of steroidogenic regulation. Thus, VIP could be involved in the tonic control of steroid secretion whilst PACAP, with its greater potency on catecholamine release, may regulate catecholamine and steroid secretion under increased levels of stress. It should also be noted that some isoforms of the PACAP receptor are linked to phosphoinositide turnover, unlike existing VIP-specific receptors. Spengler *et al* (1993) observed that the *hop* isoform of the PAC1 receptor, which predominates in the rat adrenal gland, stimulates both adenylate cyclase activation and phosphoinositide turnover. Thus, if specific receptors for both peptides are present in the adrenal cortex, the types of intracellular signalling systems activated may result in different patterns of steroid regulation between VIP and PACAP.

It may even be proposed that, in some species, VIP has a relatively minor function in the adrenal medulla compared with PACAP. Indeed, the PAC1 receptor has also been implicated in the regulation of chromaffin cell proliferation (Frodin *et al*, 1995). One speculation is that activation of PAC1 receptors on chromaffin cells and ganglion cells could result in the release of VIP as well as catecholamines. The action of VIP in the adrenal would then become a predominantly cortical one. However, variations between species cannot be ruled out. Bodart *et al* (1997) recently indicated PAC1 receptor-mediated aldosterone secretion from cultured bovine zg cells. The types of VIP / PACAP receptors present in the human adrenal have yet to be defined. This study found that VPAC receptors, probably VPAC1 receptors, are predominant on adrenocortical cells of human origin.

7.0 Steroid responses of H295R cells to a range of putative paracrine and neuroendocrine agonists

7.1 Introduction

Other agonists, aside from VIP, have been suggested to have potential paracrine and neuroendocrine actions on steroidogenesis. Many of these have established indirect effects on adrenal steroid secretion. The discovery of these substances within the adrenal, either within nerves or chromaffin cells, in parallel with the demonstration of both neural and chromaffin tissue in the adrenal cortex, provides indirect evidence for an action for these substances on steroidogenesis. The investigation of direct effects of these substances on human adrenocortical cells has been hampered by the lack of easily available human adrenal glands for *in vitro* studies, whilst data obtained *in vivo* is often hard to dissect in terms of direct and indirect actions.

In this chapter, the steroid responses of the H295R human adrenocortical tumour cell-line was used to screen a number of putative paracrine and neuroendocrine agonists for steroidogenic activity. The agonists examined were atrial natriuretic peptide / hormone (ANP), adenosine 5'-triphosphate (ATP), 5-hydroxytryptamine (5-HT / serotonin), arginine vasopressin (VP), acetylcholine (ACh) and noradrenaline (NA). These factors are introduced below with existing evidence for paracrine or neuroendocrine effects on the adrenal cortex.

7.1.1 Atrial natriuretic peptide / hormone (ANP)

ANP was first isolated from atrial myocytes by de Bold in 1982 and has been shown to exert various actions, including vasodilation and natriuresis in the kidney and inhibition of the renin-angiotensin-aldosterone system (for review see Cantin & Genest, 1985). Many of these effects antagonise the actions of AII.

More recently, ANP has been detected in a number of extra-atrial tissues such as the brain, lung and the adrenal gland. Lee *et al* (1994) detected ANP-

immunoreactivity and ANP mRNA in the adrenal medulla of humans. In rats and guinea-pigs, ANP was localised further to the medullary chromaffin cells (McKenzie *et al*, 1985; Wolfensberger *et al*, 1995).

ANP has been well documented as having a direct inhibitory effect on basal and AII-, potassium- and ACTH- stimulated aldosterone secretion from mammalian zgc cells (reviewed by Ganguly, 1992). These effects have been detected *in vivo* and *in vitro* and can be attributed to both a humoral action and a paracrine action of ANP on adrenocortical cells.

The action of ANP on zfr cells is more controversial. In most studies, ANP did not inhibit steroid secretion from inner zone cells (Atarashi *et al*, 1985; Campbell *et al*, 1985; Kudo & Baird, 1984). However, specific receptors have been detected in bovine zfr cells (Higuchi *et al*, 1986b) and rat zfr cells (Mulay *et al*, 1995) and ANP depressed basal glucocorticoid secretion from cultured inner-zone cells from humans (Higuchi *et al*, 1986a; Naruse *et al*, 1987; Carr & Mason, 1988) and cattle (DeLean *et al*, 1984). It has been postulated that ANP may also act indirectly on steroid synthesis by inhibiting the medullary release of catecholamines in cattle (Fernandez *et al*, 1992; Babinski *et al*, 1995) and rats (Vatta *et al*, 1994) via specific receptors found on medullary cells.

7.1.2 Adenosine 5'-triphosphate (ATP)

ATP is stored in high concentrations in medullary chromaffin cell secretory granules (Sillero *et al*, 1994) and is co-released with catecholamines upon stimulation. A paracrine action of ATP on adrenocortical steroidogenesis from bovine inner-zone cells has been demonstrated (Hoey *et al*, 1994) and attributed to a direct activation of the nucleotide receptor / P2y purinoceptor (Hoey *et al*, 1994 / Kawamura *et al*, 1991) and an increased turnover of membrane phosphoinositides with a resultant increase in intracellular Ca^{2+} concentration (Hoey *et al*, 1994). ATP treatment was also associated with a small but significant cAMP response (Hoey *et al*, 1994).

In addition, supramaximal electric field stimulation resulted in release of ATP from rat adrenal capsule-glomerulosa preparations and ATP has been shown to stimulate steroid secretion *in vitro* (Juranyi *et al*, 1997). ATP-containing noradrenergic nerve endings have been demonstrated in close proximity to rat zg cells and have been shown to be capable of the co-release of ATP with NA and dopamine (Szalay *et al*, 1998).

7.1.3 5-hydroxytryptamine / serotonin (5-HT)

5-HT is known to stimulate adrenocortical steroidogenesis indirectly through an effect on the HPA axis. Specifically, 5-HT causes the release of CRH from the hypothalamus (Fuller *et al*, 1992, for review) and ACTH from the anterior pituitary (Davies *et al*, 1992).

In addition, there is evidence that 5-HT also exerts a direct action on the adrenal cortex. Plasma levels of 5-HT are very low, as a result of an active platelet uptake and storage mechanism (Zinner *et al*, 1983), so that a direct action on the adrenal cortex would require local release.

5-HT has been detected immunocytochemically in the adrenal medulla of rats (Holzwarth *et al*, 1984; Holzwarth & Brownfield, 1985), mice (Fernandez-Vivero *et al*, 1993) and pigs (Kong *et al*, 1989), though evidence to date has failed to demonstrate that human chromaffin cells contain 5-HT (Lefebvre *et al*, 1992). Instead, 5-HT appears to be restricted to intra-adrenal mast-like cells (Lefebvre *et al*, 1992, 1996). In addition, serotonergic medullary tissue / nerve fibres have been detected within the adrenal cortex of mice (Fernandez-Vivero *et al*, 1993).

5-HT has been shown to stimulate steroid secretion from both zg and zfr cells in humans, requiring the activation of 5HT₄ receptors (Lefebvre *et al*, 1992, 1993, 1996). In humans, this effect is associated with an increase in Ca²⁺ influx (Contesse *et al*, 1996) and stimulation of adenylate cyclase (Lefebvre *et al*, 1992).

7.1.4 Arginine-vasopressin (VP)

VP has been shown to modulate adrenal function by an action on the HPA axis. VP is synthesised in the hypothalamus, found co-localised with corticotropin-releasing hormone (CRH) in parvocellular neurons of the paraventricular nucleus, and is stored and released from the anterior pituitary. In addition to its function as a modulator of cardiovascular homeostasis, involving antidiuretic actions in the kidney, VP stimulates the release of ACTH from the anterior pituitary (Antoni, 1986).

In addition to these indirect, humoral actions of VP on steroid secretion, a direct effect on adrenocortical cells has been reported. VP stimulated hypertrophy and hyperplasia of the zg in hypophysectomised rats (Payet *et al*, 1979) and cell growth in primary cultures of zg cells (Payet *et al*, 1984; Hinson *et al*, 1987) (reviewed by Gallo-Payet & Guillon, 1998). These growth effects appeared to involve a VP-specific receptor because growth of rat zg was suppressed following prolonged infusion with a V_1 -receptor antagonist (Mazzochi *et al*, 1993b).

VP also stimulates catecholamine release from chromaffin cells via a V_{1b} -receptor mediated mechanism (Gallo-Payet & Guillon, 1998), raising the possibility of an indirect stimulation of steroidogenesis.

VP has been shown to increase aldosterone secretion from dispersed rat zg cells in primary culture (Payet & Lehoux, 1982; Hinson *et al*, 1987), by activation of a V_{1a} -receptor (Gallo-Payet *et al*, 1986, 1991) and subsequent breakdown of membrane phosphoinositides (Gallo-Payet *et al*, 1986; Enyedi *et al*, 1988). In humans, VP similarly increased aldosterone secretion but also stimulated cortisol production by a direct receptor-mediated action (Guillon *et al*, 1995; Perraudin *et al*, 1993). VP also stimulated cortisol secretion, by activation of phospholipase C and an increase in intracellular Ca^{2+} in bovine zfr cells (Bird *et al*, 1990a; Walker *et al*, 1991). VP did not appear to directly stimulate glucocorticoid secretion in rats (Gallo-Payet *et al*, 1986), a species-specific effect that has been theoretically attributed to the lack of an active phosphoinositide second messenger system in rat

zfr cells. Specific VP receptors of the V_{1a} subtype have been located on both zg and zfr cells in human and zfr cells in cattle (Guillon *et al*, 1995).

It has been postulated that the direct action of VP on the adrenal cortex is a paracrine action of the peptide, following synthesis and release from the adrenal medulla. VP immunoreactivity has been detected in the adrenal medulla of humans, cattle and rats (Ang & Jenkins, 1984; Nussey *et al*, 1987) and further localised to the medullary chromaffin cells (Ravid *et al*, 1986; Hawthorn *et al*, 1987). Perraudin *et al* (1993) detected the presence of VP-containing cells in the medulla which were also scattered throughout the cortex. In addition, Guillon *et al* (1995) reported VP release from human chromaffin cells.

7.1.5 Acetylcholine (ACh)

ACh released from sympathetic nerve endings within the adrenal medulla stimulates the release of catecholamines from chromaffin cells by activation of a nicotinic ACh receptor. Acetylcholinergic nerve fibres have also been detected within the adrenal cortex of several species (section 1.6.4.1a)). In 1952, Okinaka *et al* showed that stimulation of the splanchnic nerve caused an increase in corticosteroid secretion from the stimulated adrenal, but not from the contralateral adrenal, suggesting that the steroidogenic effect of nerve stimulation was independent of humourally borne agonists. Perfusion of isolated calf adrenals with ACh resulted in increased 17-hydroxylated steroid secretion (Rosenfield, 1955). This evidence would support a paracrine action of ACh on the adrenal cortex.

Indeed, ACh has been shown to stimulate the secretion of cortisol and aldosterone from isolated bovine adrenocortical cells (Kojima *et al*, 1986; Walker *et al*, 1990) and perfused rat adrenals (Porter *et al*, 1988). In bovine zfr cells, this was attributed to a direct activation of the M3-muscarinic receptor (Walker *et al*, 1990; Clyne *et al*, 1994) followed by an increase in membrane phosphoinositide breakdown (Clyne *et al*, 1992) and mobilisation of intracellular Ca^{2+} (Clyne *et al*, 1995).

7.1.6 Noradrenaline (NA)

The majority of catecholaminergic nerves detected within the adrenal cortex are noradrenergic (Vizi *et al*, 1992, 1993), located mainly in the subcapsular-zg region. Some of these neurons have endings in close contact with zg cells (Vizi *et al*, 1992). Supramaximal electric field stimulation in superfused rat adrenal capsule-glomerulosa preparations resulted in the release of noradrenaline (Juranyi *et al*, 1997). The non-synaptic nature of the nerve terminals (Szalay *et al*, 1998) favours a paracrine modulation of zg cell function involving diffusion of noradrenaline from the nerve ending to the zg cell and subsequent activation of β -adrenoceptors. Chromaffin cells within the medulla and also the cortex (section 1.6.4.2) also provide a potential source of noradrenaline which may regulate steroidogenesis in a paracrine manner. The major source of the catecholamine adrenaline (Adr) appears to be the medulla. Some chromaffin cells contain both NA and Adr, whilst some are exclusively adrenergic or noradrenergic.

Although noradrenaline influences steroidogenesis by regulating blood flow through the adrenal cortex (Vinson *et al*, 1987), evidence exists for a direct action of noradrenaline on steroid secretion. *In vitro* studies in cattle and rats demonstrated stimulation of glucocorticoids, aldosterone and androstenedione secretion via β_1 -adrenergic receptor activation (Lightly *et al*, 1990; Pratt *et al*, 1985).

The 4 h and 24 h steroid responses of the H295R cell-line to each of these agonists were examined. In addition, the 4 h responses of the cells to these agonists following pretreatment of the cells with AII or forskolin were investigated. This was based on findings (in chapter 3.0) which demonstrated an increased steroidogenic capacity of the cells and also an alteration in the steroid phenotype of the cells following pretreatment with AII or forskolin. Thus, the effects of each of these agonists on relatively zg-like cells and zfr-like cells could be studied.

7.2 Results

7.2.1 Cortisol response to 4 h treatment with ANP, ATP, 5-HT, VP, ACh and NA in H295R cells

Figure 7.1 shows the cortisol responses to short term (4 h) treatment with ANP, ATP, VP, 5HT, ACh and NA. Cells were treated with forskolin as a positive control.

4 h ANP (10^{-7} M) treatment caused a 40 (± 4.5) % decrease in cortisol secretion, relative to basal levels (n=3 individual experiments, $P < 0.05$ in 3/3 experiments, calculated from triplicate incubations within each experiment, of which $P < 0.01$ in 2 of these).

4 h treatment with ATP (10^{-4} M), 5-HT (10^{-6} M), VP (10^{-8} M), ACh (10^{-4} M) or NA (10^{-6} M) did not affect cortisol levels with respect to basal secretion ($P \geq 0.05$ in n=4 experiments, except for VP where n=3 experiments. P-values were calculated from triplicate incubations within each experiment).

7.2.2 Effect of pretreatment of cells with AII or forskolin on subsequent 4 h steroid responses to ANP, ATP, 5-HT, VP, ACh and NA in H295R cells

Figure 7.2 shows the effects of the different pretreatment conditions on steroid responses to subsequent 4 h treatment with ANP, ATP, 5-HT, VP, ACh and NA. Cells were treated with forskolin as a positive control.

In “control” cells (that is, no pretreatment with AII or forskolin), 4 h treatment with ANP (10^{-7} M) resulted in a significant decrease in cortisol secretion (45 (± 4) %, n=3 individual experiments, $P < 0.02$ in 3/3 experiments, calculated from triplicate incubations within each experiment) and corticosterone secretion (63 (± 9) %, n=3 individual experiments, $P < 0.01$ in 3/3 experiments, calculated from triplicate incubations within each experiment), but had less effect on androstenedione

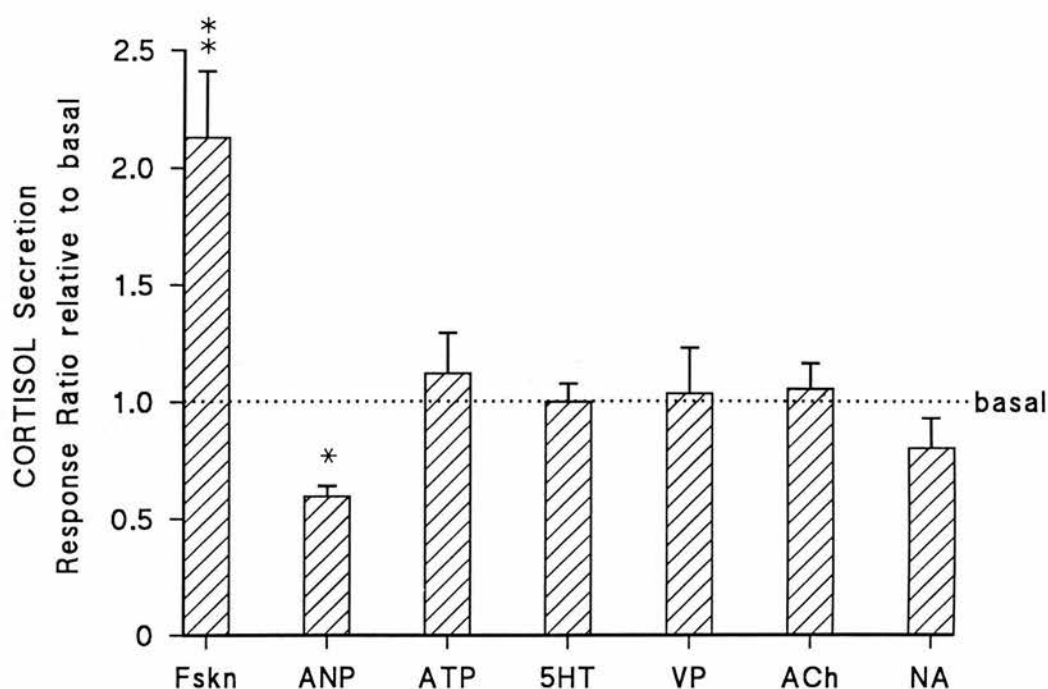


Figure 7.1

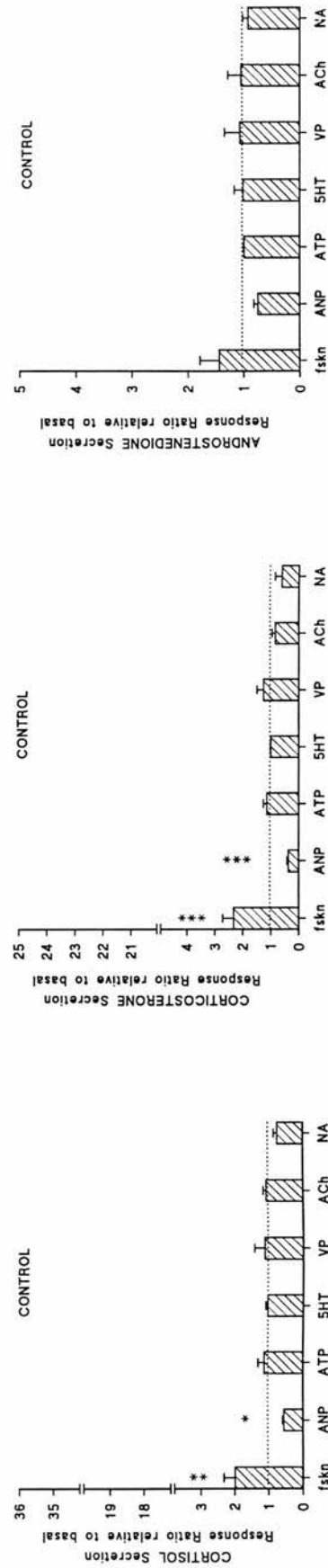
*Cortisol secretion in response to short-term (4 h) treatment with various putative paracrine and neuroendocrine agonists. Cells were treated with medium alone (basal, dotted line) or medium containing the agonist to be tested (see Key below) for 4 h. After this time, medium was removed and assayed for cortisol content by RIAs. The results from n=4 separate experiments (n=3 for ANP and VP) have been combined and are presented as mean Response Ratios (response to stimulus / basal secretion) (\pm s.d.). Statistically significant changes in secretion compared with basal secretion within individual experiments are noted by **P<0.02 and *P<0.05, in 4/4 experiments for fskn; in 3/3 experiments for ANP.*

Key: fskn=forskolin, 10^{-5} M; ANP=atrial natriuretic peptide, 10^{-7} M; ATP=adenosine triphosphate, 10^{-4} M; 5HT=5-hydroxytryptamine/serotonin, 10^{-6} M; VP=vasopressin, 10^{-8} M; ACh=acetylcholine, 10^{-4} M; NA=noradrenaline, 10^{-6} M;

Figure 7.2

Effect of 96 h AII or forskolin pretreatment of H295R cells on the subsequent 4 h cortisol, corticosterone and androstenedione responses to various putative paracrine and neuroendocrine steroidogenic agonists. Cells were pretreated with growth medium containing either AII (10 nM, “AII pretreatment”), forskolin (10 μ M, “Forskolin pretreatment”) or no added agonist (“control”) for 96 h. Medium and pretreatment agonists were replaced every 24 h. After 96 h pretreatment, cells were washed thoroughly and incubated with either growth medium alone (basal, dotted line) or medium containing an agonist (see *Key* below) for 4 h. After this time, medium was removed and assayed for cortisol, corticosterone and androstenedione by RIA. Steroid data was corrected for total cellular protein. Combined data from n=3 separate experiments is presented as mean (\pm s.d.) Response Ratios (response to stimulus / basal secretion). Basal secretion is represented by the dotted line. Statistically significant changes in steroid secretion with respect to basal secretion are noted by * $P<0.05$, ** $P<0.02$ and *** $P<0.01$, within each experiment performed. For “AII pretreatment” and “Forskolin pretreatment” results, see next page.

Key: fskn = forskolin, 10⁻⁵ M; ANP = atrial natriuretic peptide, 10⁻⁷ M; ATP = adenosine 5'-triphosphate, 10⁻⁴ M; 5HT = 5-hydroxytryptamine / serotonin, 10⁻⁶ M; VP = arginine vasopressin, 10⁻⁸ M; ACh = acetylcholine, 10⁻⁴ M; NA = noradrenaline, 10⁻⁶ M.



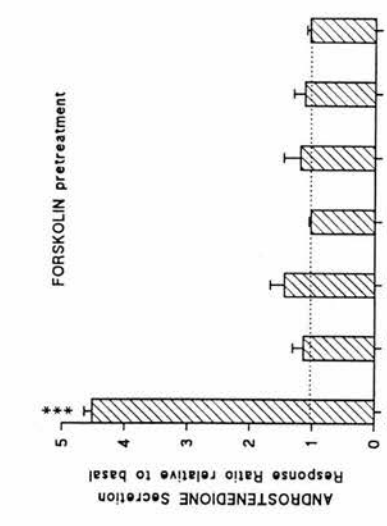
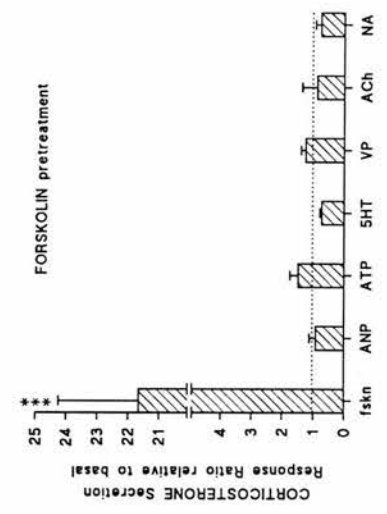
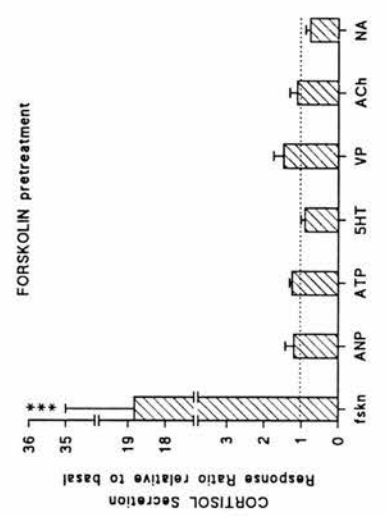
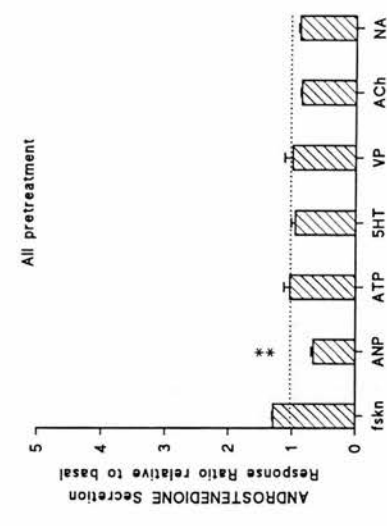
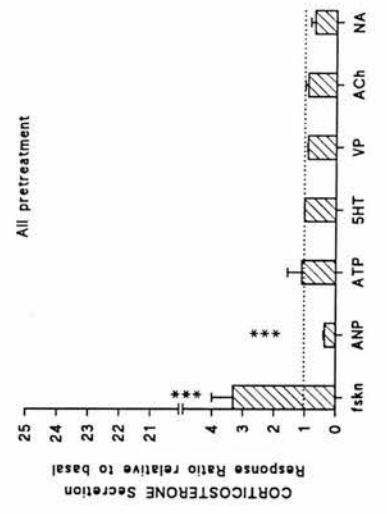
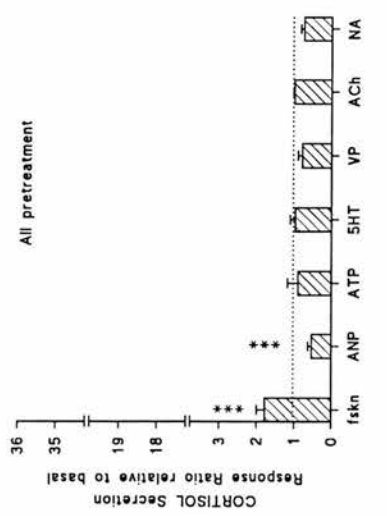


Figure 7.2 continued: see previous page for figure legend.

Key: fskn = forskolin, 10^{-5} M; ANP = atrial natriuretic peptide, 10^{-7} M; ATP = adenosine triphosphate, 10^{-4} M; 5HT = 5-hydroxytryptamine / serotonin, 10^{-6} M; VP = arginine vasopressin, 10^{-8} M; Ach = acetylcholine, 10^{-4} M; NA = noradrenaline, 10^{-6} M.

production (a decrease of 15 (± 12) %, $n=3$ individual experiments, $P<0.02$ in 2/3 experiments, calculated from triplicate incubations within each experiment), from “control” cells.

4 h treatment with ATP (10^{-6} M), 5-HT (10^{-6} M), VP (10^{-8} M) or ACh (10^{-4} M) had no effect on secretion of cortisol, corticosterone or androstenedione from “control” cells ($P\geq 0.05$ for 3/3 individual experiments, calculated from triplicate incubations within each experiment).

Following 96 h pretreatment of H295R cells with AII (10 nM), the steroid responses to the substances tested were similar to those obtained from “control” cells. ANP (10^{-7} M) suppressed secretion of all 3 steroids (decreases of 47 (± 17) %, 64 (± 8) % and 34 (± 7) % for cortisol, corticosterone and androstenedione respectively ($n=3$ experiments for all three steroids. Within each experiment, $P<0.01$ for cortisol and corticosterone data and $P<0.02$ for androstenedione data, calculated from triplicate incubations). ATP (10^{-6} M), 5-HT (10^{-6} M), VP (10^{-8} M) or ACh (10^{-4} M) had no effect on secretion of cortisol, corticosterone or androstenedione from “AII-pretreated” cells ($P\geq 0.05$ for 3/3 experiments, calculated from triplicate incubations performed in each experiment).

Following forskolin pretreatment, none of the agonists tested (apart from the positive control, forskolin (10^{-5} M), altered steroid secretion with respect to basal. In particular, forskolin pretreatment abolished the ANP-induced suppression of steroid secretion seen in “control” and “AII-pretreated” cells ($P\geq 0.05$ with respect to basal secretion of each steroid in 3/3 separate experiments, triplicate incubations performed in each) (see *Figure 7.2*).

7.2.3 Steroid responses to 24 h treatment with ANP, ATP, 5-HT, VP, ACh and NA in H295R cells

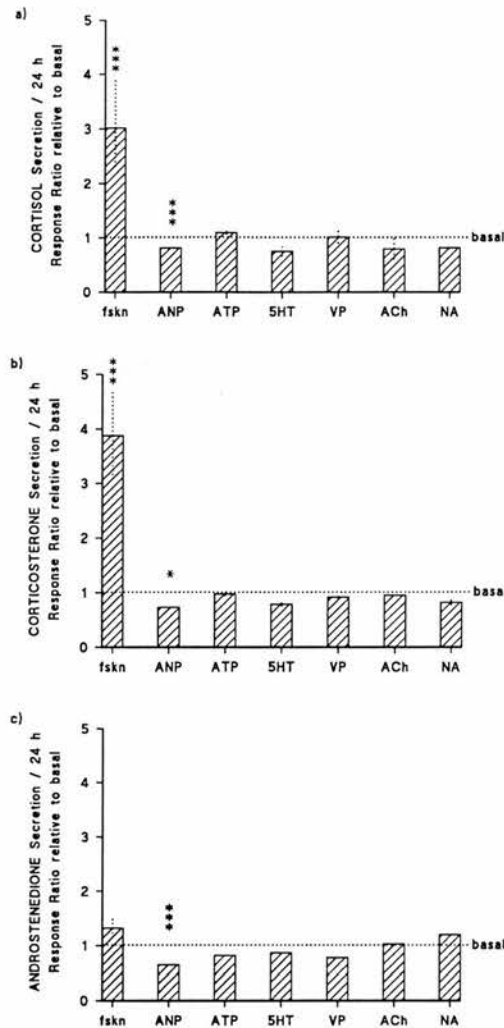
Only ANP (and the positive control agonist, forskolin) caused changes in steroid secretion after short-term (4 h) treatment. The H295R cells were treated with

ANP, ATP, 5-HT, VP, ACh and NA for 24 h to see if longer exposure to these agonists would elicit a cortisol response.

Figure 7.3 shows the cortisol, corticosterone and androstenedione responses to 24 h treatment with ANP, ATP, 5-HT, VP, ACh and NA. Cells were also treated with forskolin as a positive control.

24 h treatment with ANP (10^{-7} M) significantly suppressed secretion of all 3 steroids, with respect to basal secretion, with decreases of 19% for cortisol, 27% for corticosterone and 35% for androstenedione ($P < 0.01$ for cortisol and androstenedione data and $P < 0.05$ for corticosterone data, calculated from triplicate incubations in a single experiment).

24 h treatment with either ATP (10^{-4} M), 5-HT (10^{-6} M), VP (10^{-8} M), ACh (10^{-4} M) or NA (10^{-6} M) had no significant effect on cortisol, corticosterone or androstenedione secretion ($P \geq 0.05$, calculated from triplicate incubations performed in each of 2 separate experiments) (see *Figure 7.3*).



Key: fskn = forskolin, 10^{-5} M (positive control); ANP = atrial natriuretic peptide, 10^{-7} M; ATP = adenosine triphosphate, 10^{-4} M; 5HT = 5-hydroxytryptamine / serotonin, 10^{-6} M; VP = vasopressin, 10^{-8} M; ACh = acetylcholine, 10^{-4} M; NA = noradrenaline, 10^{-6} M.

Figure 7.3

Secretion of a) cortisol, b) corticosterone and c) androstenedione in response to 24 h treatment with various putative steroidogenic agonists. Cells were treated with medium alone (basal, dotted line) or medium containing an agonist (see *Key*) for 24 h. After this time, medium was removed and assayed for steroid content by RIAs. Results have been combined and are presented as mean (\pm range, i.e. max-min) Response Ratios (response to stimulus / basal secretion) from $n=2$ individual experiments for cortisol and corticosterone, except ANP (where $n=1$ experiment), triplicate incubations performed in each experiment. Androstenedione data was obtained from $n=1$ experiment, triplicate incubations performed, for all agonists. Statistically significant changes in secretion compared with basal secretion within each individual experiment performed are noted by * $P<0.05$ and *** $P<0.01$.

7.3 Discussion

The steroidogenic actions of various putative paracrine and neuroendocrine agonists (ANP, ATP, 5-HT, VP, ACh and NA) were investigated in the H295R human adrenocortical tumour cell-line. Only ANP had any significant and reproducible effect on steroidogenesis in H295R cells. The steroid responses of the cells to forskolin (10^{-5} M) were similar to those presented in chapter 3.0.

Short-term (4 h) treatment with ANP resulted in decreases in basal cortisol and corticosterone secretions (45 (± 4) % and 63 (± 9) % respectively, $n=3$ experiments for both steroids). Androstenedione secretion was decreased to a lesser degree (15 (± 12) %, $n=3$ experiments). This inhibitory effect of ANP agrees with findings following ANP infusion into healthy human volunteers, which resulted in an inhibition of steroid production from all three zones of the cortex, including aldosterone, cortisol and DHEA (Nawata *et al*, 1991).

Of the three steroids measured in H295R cells, corticosterone was inhibited to the greatest extent by ANP. ANP is a well-documented inhibitor of aldosterone synthesis (section 7.1.1 and Ganguly (1992) for review) and the decrease in corticosterone secretion implies that ANP inhibits the mineralocorticoid pathway at an earlier stage than corticosterone production in these cells. This would be consistent with evidence that ANP exerts its action at a very early stage of steroid synthesis involving cholesterol translocation into mitochondria and conversion to pregnenolone (Goodfriend *et al*, 1984). It would be interesting to examine the effects of ANP on StAR expression in H295R cells. ANP also significantly suppressed P450_{scc} mRNA in ACTH-stimulated cultured bovine adrenal cells (Nawata *et al*, 1991). In another study, ANP was shown to inhibit both early and late steps in the steroid biosynthesis pathway (Campbell *et al*, 1985).

Androstenedione secretion was unaffected by short-term (4 h) treatment with ANP. This finding is unexpected if, as has been postulated, androstenedione secretion is extremely cAMP dependent in this cell-line (chapter 3.0). ANP caused decreases in cAMP and steroidogenesis in Y1-cells stimulated with ACTH (Heisler

et al, 1989) and in dispersed adrenal capsular tissue (Matsuoka *et al*, 1984, 1985). If ANP was acting by inhibition of adenylate cyclase, an inhibition of androstenedione secretion would be predicted.

A previous study on H295R cells showed that ANP failed to inhibit forskolin-stimulated aldosterone secretion (Bodart *et al*, 1996). In the present study, forskolin pretreatment of cells abolished the ANP-induced inhibition of basal steroid secretion. One possible explanation would be a reduction in the number of ANP receptors expressed following forskolin pretreatment of the H295R cells. Of relevance is the observation that the density of cell-surface ANP binding sites is lower on zfr cells compared with zg cells, as demonstrated by autoradiography (Nunez *et al*, 1990), since pretreatment of H295R cells with forskolin results in a zfr-like steroid phenotype (chapter 3.0). In addition, the direct effects of ANP on zfr cells have been controversial (section 7.1.1).

It has been postulated that ANP can lower cAMP levels in several ways. In addition to receptor-activated G-protein inhibition of adenylate cyclase (Anand-Srivastava *et al*, 1984, 1985, 1987), ANP caused increases in intracellular cGMP and activated a cGMP-dependent PDE, which subsequently lowered cAMP levels in primary bovine zg cells (MacFarland *et al*, 1991). Thus, basal levels of cAMP may be reduced. Bodart *et al* (1996) reported ANP-stimulated increases in cGMP in H295R cells. However, if basal cAMP levels were increased following forskolin pretreatment of H295R cells, it is conceivable that the subsequent suppression by ANP may not be sufficient to attenuate basal steroidogenesis in these pretreated cells. Indeed, ANP only reduced the sensitivity of zg cells to ACTH rather than completely blocking ACTH-induced steroidogenesis (Ganguly, 1992). This needs to be investigated further.

Although ATP has been shown to directly stimulate cortisol secretion from bovine and rat adrenocortical cells *in vitro* (Hoey *et al*, 1994; Juranyi *et al*, 1997), this nucleotide failed to elicit a steroid response from the H295R cell-line. The true situation *in vivo* in humans is therefore unclear.

5-HT also failed to stimulate steroid secretion from H295R cells. This contrasts with studies performed on human adrenals where 10^{-7} M 5-HT directly stimulated both cortisol and aldosterone secretion (Lefebvre *et al*, 1992, 1993), an effect mediated via 5HT₄ receptor activation and an increase in cAMP formation (Lefebvre *et al*, 1992). Since the cAMP second messenger system is intact in this cell-line, the inference is that the 5HT₄ receptor is absent in H295R cells. Lefebvre *et al* (1992) observed a partial desensitisation following prolonged exposure (up to 3 h) to 5-HT on perfused human adrenocortical slices, which was postulated to be due to a down-regulation of the 5HT₄ receptor. Since the H295R cell medium, collected following 4 h exposure to 5-HT, contains the accumulated cortisol secreted during that 4 h period, any stimulation of steroid secretion during that treatment period should be detected as an increase in total cortisol content of the medium. Thus, although the H295R cells were exposed to 5-HT for 4 h, it is unlikely that the lack of response is due to desensitisation of the receptor in these cells.

Administration of the 5HT₄ receptor-specific agonist, zacopride, to human adrenocortical slices indicated that 5-HT is at least 100 times more potent at stimulating aldosterone secretion than cortisol secretion, a finding that was consistent with *in vivo* human studies where zacopride infusion resulted in an increase in plasma aldosterone but not cortisol (Lefebvre *et al*, 1993). Thus, a reason for the apparent lack of steroidogenic action of 5-HT on H295R cells may be due to the problems experienced in detecting aldosterone in these cells.

Contrary to previous studies performed on primary cultures of human adrenocortical cells (Perraudin *et al*, 1993; Guillon *et al*, 1995), VP failed to stimulate steroid secretion from H295R cells. Pretreatment of the cells to alter the steroidogenic phenotype failed to influence the lack of steroid response to VP. Grazzini *et al* (1996) recently showed that pretreatment of adrenocortical cells with the cAMP agonist, ACTH, negatively modulated VP binding and second messenger activation. Guillon *et al* (1988) also demonstrated that 2 h exposure to ACTH decreased inositol phosphate accumulation and VP-receptor binding. Thus, pretreatment of H295R cells with forskolin, whilst increasing the sensitivity of the

cells to some steroidogenic agonists, might decrease or inhibit the responsiveness of these cells to VP.

ACh and NA both failed to stimulate steroidogenesis from the H295R cell-line, in contrast to *in vitro* studies performed in other species. A species-specific difference may account for this observation. Alternatively, the effects observed in other species could be indirect, involving release of secretagogues, such as catecholamines, from interspersed chromaffin cells in the primary culture. Indeed, the effects of ACh on steroidogenesis have been attributed partly to an indirect mechanism involving increased blood flow to the adrenal (and thus increased rate of delivery of other stimulatory agonists to the cortex) and partly to release of catecholamines from the medulla, which subsequently stimulate steroid secretion via β -adrenoceptors. Studies with this human adrenocortical cell-line were unable to clarify whether ACh might have a direct action in humans *in vivo*, but would argue against this.

In conclusion, the cortisol, corticosterone and androstenedione responses to a range of potential steroidogenic agonists (selected on the basis of studies showing an effect in other systems), were investigated in the H295R cell-line. For most of the agonists tested in this study, no evidence for a direct action on adrenocortical cells of human origin was obtained when using the H295R cell-line as a model. This could be attributed to a species-specific difference. Several of the agonists tested have previously been shown to affect adrenocortical steroid production in man and yet failed to do so in this human adrenocortical cell-line. The differences in data obtained compared with previous human data may indicate indirect actions of these agonists on steroid secretion, rather than a direct effect on adrenocortical cells. Another possibility is that receptors for some agonists may have become mutated or down-regulated in these cells. However, whilst the transformed nature of the cell-line cannot be ignored, the ability of the cells to produce end-pathway adrenal steroids and the maintenance of a steroid response to the major physiological regulators of steroid synthesis (AII, K^+ , ANP) and to activators (or analogues) of the cAMP, PI

and calcium second messenger pathways (Bird *et al*, 1993, 1995b; Gazdar *et al*, 1990; Rainey *et al*, 1993) is in keeping with properties of normal adrenocortical cells and indicates that the cell-line is of use in investigating the function of putative steroidogenic agonists. Of particular interest is the ability to alter the steroid phenotype of the H295R cells towards a potentially zg-like or zfr-like phenotype by pre-incubation with AII or forskolin, respectively.

8.0 Concluding remarks

The aims of this thesis were to 1) further characterise the time-dependent effects of AII and forskolin on steroid production by H295R cells, 2) examine the possibility of altering the steroid phenotype of the H295R cell-line, 3) investigate the direct effects of VIP on steroid production from H295R cells and 4) characterise the second messenger systems and receptor types involved in VIP-stimulated cortisol production (section 1.9). Extensive evidence already existed for an indirect effect but a direct action of VIP on adrenocortical cells was still controversial. In addition, little data had been reported relating to the effects of VIP on steroidogenesis in humans.

In vitro studies, whilst not necessarily reflecting the whole physiological situation *in vivo*, are often a useful tool for obtaining an insight into possible *in vivo* mechanisms. The difficulty obtaining fresh human adrenal tissue, either normal or abnormal, has particularly hampered investigations. On this basis, the H295R cell-line was selected as a useful model for these studies because it was of human origin and had previously been shown to respond to several physiological regulators of steroidogenesis, such as AII, ACTH and K^+ (Rainey *et al*, 1994). In addition, it was the first human adrenocortical cell-line characterised as expressing the full complement of adrenal steroidogenic enzymes with the capability of secreting the major adrenocortical steroids (Gazdar *et al*, 1990).

The time-dependent effects of AII and forskolin treatment on cortisol, corticosterone and androstenedione secretion were studied. The profile of steroids secreted differed for the two agonists and was dependent on the exposure time to each agonist (chapter 3.0). Studies performed in this thesis also demonstrated that the steroid phenotype of the cell-line could be altered by pretreatment with AII or forskolin, resulting in relatively zg-like and zfr-like phenotypes, respectively (chapter 3.0). These findings make the H295R cell-line a potentially useful model for investigating actions in zg-like and zfr-like cells, particularly since primary cultures of adrenocortical cells quickly revert to a de-differentiated state (Hornsby & Crivello, 1983) and often lose steroidogenic capacity altogether (Walker *et al*, 1988; Williams

et al, 1989). The fact that the cell-line can be subcultured also obviates the necessity for a continuous source of new tissue and overcomes problems related to the variability in results between primary cultures of different tissue origin. The cell-line would also be potentially useful in investigating factors involved in zonation of the adrenal cortex.

Against this background, the H295R cell-line was used to investigate a possible direct action of VIP in human adrenocortical cells. The chronic and short-term effects of VIP on the production of steroids from the three major pathways were examined. Chronic VIP treatment stimulated production of cortisol and corticosterone, but not androstenedione, in a time-dependent manner. VIP was more potent in stimulating cortisol secretion than corticosterone production, both acutely and chronically, as evidenced by the increase in the ratio of cortisol / corticosterone secretion with time (chapter 4.0). The chronic effects of VIP could involve an upregulation of steroidogenic enzymes, in particular P450c17. This would account for the greater effect of VIP on cortisol secretion rather than corticosterone secretion.

Pretreatment of H295R cells with VIP increased cortisol response to a subsequent 4 h treatment with VIP, further suggesting an effect of chronic VIP treatment on steroidogenic enzyme expression and / or VIP receptor expression (chapter 4.0). Studies into the effect of VIP pretreatment on the subsequent 4 h VIP-induced cortisol / corticosterone and cortisol / androstenedione secretion ratios may provide clues as to which steroid pathway chronic VIP treatment preferentially stimulates. Further experiments to investigate the effect of chronic VIP treatment on the expression of key steroidogenic enzymes, for example, P450c17 and 3 β -HSD, would be of interest.

The effects of short-term VIP treatment on steroid production from cells with a zg-like phenotype and a zfr-like phenotype were also examined (that is, following pretreatment of the cells with either AII or forskolin, respectively, for 96 h). An interesting observation was that VIP stimulated androstenedione secretion from zfr-like cells (that is, forskolin-pretreated cells) in addition to increasing production of cortisol and corticosterone.

Thus, it is interesting to speculate whether VIP is the putative CASH (section 1.4.3). Recent evidence has strengthened the argument for the existence of an androgen-stimulating hormone in the regulation of adrenal androgen production, rather than intra-adrenal factors such as exposure of the inner cortex to high concentrations of cortisol (Fearon *et al*, 1998). One proposed candidate for CASH is a pituitary-derived factor of, so far, uncertain identity (Brubaker *et al*, 1982; Hung *et al*, 1988; Parker, 1991, review). Immunoreactive VIP is released from perfused rat pituitaries and cultured rat pituitary cells (Calvo *et al*, 1990; Carretero *et al*, 1995) and VIP gene expression has been detected in the pituitary (Arnaout *et al*, 1986; Chew *et al*, 1992). However, VIP levels in peripheral blood are relatively low (approximately 50 pg / ml (Said & Porter, 1979 Cugini *et al*, 1991), whereas concentrations of VIP in hypophyseal portal blood are 19 times higher (Said & Porter, 1979)) and VIP is predominantly a neuroendocrine transmitter. Therefore, a direct stimulation of adrenal androgen secretion by VIP would probably be a neuroendocrine or paracrine event. The presence of numerous VIPergic nerves in the zr region of the adrenal cortex as well as within medullary chromaffin cells would allow for a paracrine / neuroendocrine action of VIP on zr adrenocortical cells (sections 1.6.4.1c and 1.6.4.2b)). Androstenedione secretion in response to VIP has also been reported from perfused porcine adrenal and cultured human adrenal cells (Bornstein *et al*, 1993, 1996). Stimulation of androgen secretion was found to be highly cAMP-dependent in this cell-line (chapter 3.0) and VIP was subsequently shown to increase cAMP production in these cells (chapter 5.0). However, whilst VIP appears to regulate adrenal androgen secretion, the question as to whether VIP is a major putative CASH requires further investigation.

To investigate in detail the second messenger systems activated by VIP in H295R cells, the measurements focussed upon cortisol production. A concentration-dependent increase in cAMP accumulation was observed which paralleled the increase in cortisol secretion. Changes in cGMP accumulation and PI turnover were not detected (chapter 5.0). Thus, it appears that VIP stimulates steroid production in a more ACTH-like manner than AII-like manner. Whether this means that VIP could

modulate adrenocortical responses to AII and ACTH *in vivo* in different ways is unclear.

VIPergic innervation to the zg appears to be predominantly extrinsic in origin and may be associated with splanchnic innervation, whilst the VIPergic innervation of the zfr appears to arise from an intra-adrenal source, with VIP-containing ganglion cells within the medulla (Hokfelt *et al*, 1981; Holzwarth *et al*, 1987; Oomori *et al*, 1994; Hinson & Kapas, 1996). These two different sources of VIP innervation may allow a differential regulation of the different zones of the cortex by VIP. To speculate, it is possible that VIPergic innervation of the zg may be predominantly involved in the regulation of adrenal blood flow since VIP has been shown to increase perfusion flow rate through the adrenal (Hinson *et al*, 1994a) and many VIPergic nerves have been found in close apposition to blood vessels in the capsular-zg region (Oomori *et al*, 1994). In addition, the sub-capsular VIPergic neural plexus may innervate the numerous chromaffin cells detected within the zg region.

Studies reported here also show that VIP and PACAP stimulate cortisol secretion with similar potency from H295R cells, consistent with activation of a VPAC receptor rather than a PAC1 receptor (chapter 6.0). RT-PCR analysis of H295R cell mRNA and pharmacological studies with VPAC1 receptor antagonists and a VPAC2 receptor superagonist provided evidence for an effect mediated by a VPAC1 receptor (chapter 6.0).

The importance of these findings in relation to the situation *in vivo* need to be assessed. The problems with extrapolating data from *in vitro* studies are difficult enough since cells in monolayer do not exhibit the same architecture as in intact adrenal and lack the continuous washout and replenishment of substances by the blood supply, for example, ACTH. Nevertheless, *in vitro* studies do provide important information which can be coupled to existing *in vivo* data. Although the H295R cell-line exhibits a transformed phenotype and the cells differ from normal adrenocortical cells in some respects, many of the cell properties are similar to normal, non-transformed adrenocortical cells (section 1.8).

On the basis that the findings obtained within this thesis reflect the *in vivo* situation, a physiological rationale for a direct action of VIP on adrenocortical steroidogenesis must be sought. The acute release of steroids from the adrenal cortex is a component of the stress response, whether that stress be of a haemodynamic nature, altered electrolyte status or in response to cold, hypoxia, inflammation or injury, for example. This response requires activation of several systems including the sympatho-adrenal system, the immune system and the HPA axis. VIP has been implicated in the regulation of all these systems.

In particular, VIP may regulate the HPA axis at all levels. Thus, it stimulates the release of CRH from the hypothalamus and potentiates the effect of CRH on ACTH release from the pituitary (Alexander *et al*, 1994). VIP mRNA has been detected in the anterior pituitary (Lam, 1991) and VIP may stimulate ACTH directly from anterior pituitary corticotrophs, though this effect may be more significant in pathological conditions, such as ACTH-secreting pituitary adenomas (Watanobe & Takamura, 1994; Oliva *et al*, 1982).

In the adrenal, VIP directly regulates blood flow. Hinson *et al* (1994a) demonstrated an increase in perfusion flow rate in perfused rat adrenals and infusion of VIP into functionally hypophysectomised calves also resulted in increased adrenal blood flow (Bloom *et al*, 1987). An increase in blood flow indirectly stimulates steroidogenesis (Vinson *et al*, 1987). Another possibility is that VIP might be implicated in the stress-related release of stored steroids within the adrenal as a result of vascular damming (section 1.6.3). CRH has been suggested to be involved in release of adrenal venous congestion (Bassett & West, 1997; section 1.6.3). However, VIP could also be a candidate. A source of VIP exists in the cortico-medullary boundary (Hokfelt *et al*, 1981; section 1.6.4.1c) where a vascular dam is thought to exist and VIP is a potent vasodilator (Said & Mutt, 1970; Lee *et al*, 1984; section 1.7). Levels of VIP increase within the venous outflow following stimulation of the splanchnic nerve (Bloom *et al*, 1988; section 4.1.1) and glucocorticoid secretion is increased in the presence of VIP if the gland architecture is intact (Hinson *et al*, 1992; Holzwarth *et al*, 1987; Bernet *et al*, 1994a; section 4.1.1). The

release of dammed steroids by VIP in certain stress conditions may also explain the apparent VIP-mediated increase in adrenocortical sensitivity to ACTH often observed in *in vivo* situations (Bloom *et al*, 1987, Edwards & Jones, 1993b), though an actual increase in ACTH sensitivity of adrenocortical cells is not excluded.

Other actions of VIP in the adrenal could include a growth effect on adrenocortical cells. Whilst a growth action on zfr cells has not been shown, VIP does appear to regulate the growth and secretory capacity of rat zg (Rebuffat *et al*, 1994).

The indirect effect of VIP on adrenocortical steroidogenesis, involving catecholamine release from chromaffin cells, is now well documented though it is unclear whether this effect is more prominent in one zone than another.

With VIP involved in so many different levels of regulation of adrenocortical steroidogenesis, a key question is whether or not a direct action of VIP exists at the level of the adrenocortical cell. The recent discovery of VIP receptors in the rat zg supports such a possibility (Cunningham & Holzwarth, 1989; Hinson *et al*, 1996; Hinson & Kapas, 1997). Moreover, these receptors appear to be upregulated by low sodium status (Hinson *et al*, 1996) suggesting a more prominent direct action of VIP in response to stress than a tonic regulation of steroidogenesis. This concept would also be consistent with a VIP-mediated increase in sensitivity to ACTH, released during stress. One possibility is that VIP may maintain a level of sensitivity to ACTH under normal conditions which could be increased during stress by upregulation of VIP receptors on adrenocortical cells. Thus VIP could increase sensitivity to steroidogenic agonists, such as ACTH, and also stimulate further steroid secretion directly.

A function for VIP in the tonic maintenance of adrenocortical sensitivity is supported by the finding that infusion of a VIP antagonist caused a decrease in plasma aldosterone concentration in rats (Rebuffat *et al*, 1994). Maintenance or increase in adrenocortical sensitivity to ACTH (or other agonists) could involve putative effects of VIP on steroid enzyme levels, as hinted by the observation that VIP pretreatment of H295R cells increased the subsequent steroid responses to VIP

and forskolin (chapter 4.0). This speculation could be investigated by studying the effects of VIP on adrenocortical steroidogenic enzyme expression, for example, by Northern analysis of enzyme transcripts from adrenocortical cells. Another possibility *in vivo* is that the direct effects of VIP on adrenocortical steroidogenesis are only apparent in situations where the HPA axis is disrupted, for example, in cases of primary steroid-secreting adrenocortical tumours.

Whilst it is tempting to speculate on a physiological direct action of VIP on adrenocortical cells, the transformed nature of the H295R cell-line cannot be ignored. Thus, it is likely that the potency with which VIP stimulates steroid production from H295R cells is not reflected *in vivo*, or may only be reflected in pathophysiological conditions. A large number of tumours secrete VIP, for example, some phaeochromocytomas (Hassoun *et al*, 1984; Viale *et al*, 1985; Said, 1976) as well as some tumours of the pancreas, thyroid and lung (Said, 1976). Over-production of VIP in the adrenal medulla could contribute to conditions such as Cushing's syndrome or hyperaldosteronism associated with secreting phaeochromocytomas. In addition, some tumours exhibit increased VIP receptor expression (Reubi *et al*, 1995). An increase in VIP receptor expression in adrenal tumours could result in excessive steroid secretion, such as that observed in adrenal adenomas.

Further studies on human adrenal tissue are obviously required to determine the level of VIP receptor expression in normal human adrenal cortex. Whether or not these receptors are then upregulated by exposure human to different forms of 'stress' is of considerable interest. Measurement of the relative levels of VIP receptor expression in adrenal tumour tissue might also be informative.

Whilst this thesis was in preparation, Haidan *et al* (1998) published a report on the expression of VIP receptors in H295R cells. The findings in this thesis correlate well with the findings of this group; they found dose-dependent increases in aldosterone, cortisol and DHEA in the concentration range 10^{-9} M - 10^{-6} M, using non-pretreated cells. They determined the presence of both VPAC1 and VPAC2 receptor mRNAs by RT-PCR. In this thesis, only the VPAC1 receptor transcript was detected. However, differences in cell culture procedures could account for this. In

addition, these authors were able to measure aldosterone secretion from these cells, in contrast to the studies in this thesis.

Finally, the steroidogenic effects of a range of putative paracrine / neuroendocrine agonists were examined using the H295R cell-line to determine the existence of a direct action on adrenocortical cells (chapter 7.0). ANP caused a significant decrease in production of cortisol, corticosterone and androstenedione, which was abolished following forskolin pretreatment of H295R cells (that is, cells with a relatively zfr-like phenotype). Thus, the H295R cell-line could be a good model for investigating the mechanism of ANP action on zg-like and zfr-like cells. For example, it would be interesting to examine the relative number of ANP receptors on zg-like and zfr-like H295R cells.

The work in this thesis provides information on the H295R cell-line as a model for investigating the direct action of putative agonists on steroidogenesis factors involved in the zonation of the adrenal cortex. Also, evidence has been provided for a direct action of VIP on adrenocortical function. This could be important in increasing our understanding of mechanisms regulating adrenocortical steroidogenesis *in vivo* and perhaps aid the development of potential therapeutic agents in adrenal diseases associated with an increased response to VIP.

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COBB, V. J., WILLIAMS, B. C., MASON, J. I. AND WALKER, S. W. 1997.
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FORSKOLIN TREATMENT DIRECTS STEROID PRODUCTION TOWARDS
THE ANDROGEN PATHWAY IN THE NCI-H295R
ADRENOCORTICAL TUMOUR CELL LINE

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ABSTRACT

The human adrenocortical tumour cell line, NCI-H295, secretes steroids on the mineralocorticoid, glucocorticoid and adrenal androgen pathways. We have investigated the effects of 96 h treatment of cells in monolayer culture with either forskolin (10 μ M) (a direct activator of adenylate cyclase), angiotensin II (10 nM) or no agonist ('control') on the steroidogenic phenotype of this cell line. Androstenedione, cortisol and corticosterone secreted into the medium in response to a subsequent 4 hour treatment with angiotensin II (10nM) indicated that the steroidogenic phenotype of NCI-H295 cells changes away from 17-deoxysteroid biosynthesis towards adrenal androgen production in response to forskolin. The NCI-H295R cell line therefore serves as a useful model for investigation of the differential regulation of the steroidogenic pathways in the human adrenal cortex.

INTRODUCTION

The human adrenocortical tumour cell line, NCI-H295, produces more than 30 different steroids characteristic of mineralocorticoid, glucocorticoid and adrenal androgen pathways (1, 2). The original cell line was established as a suspension to overcome problems with fibroblast overgrowth (1). Since then, a subpopulation of cells, designated H295R cells, has been selected that retains attachment during culture (3). These cells respond to classical adrenocortical agonists such as

angiotensin II (AII), which increases aldosterone secretion (4), and forskolin, dibutyryl-cyclic AMP and, to a lesser extent, ACTH, all of which stimulate cortisol, dehydroepiandrosterone (DHEA), DHEA sulphate and androstenedione in a dose- and time- dependent fashion (4). Consistent with these observations, mRNAs encoding P450scc, 3 β HSD, P450c17; P450c21, P450c18 and P450c11 (side-chain cleavage enzyme, 3 β -hydroxysteroid dehydrogenase, 17-hydroxylase, 21-hydroxylase, 18-hydroxylase and 11-hydroxylase, respectively) have been detected in H295R cells (2,3,4,5,6) and shown to be regulated in a hormonally-sensitive manner (2,3,4,7).

To investigate this further we examined the possibility of shifting H295R cells towards a zona glomerulosa-like phenotype by chronic treatment with AII, the principal regulator of the mineralocorticoid pathway, or towards a more zona fasciculata/reticularis-like phenotype by chronic treatment with forskolin, an activator of protein kinase A.

MATERIALS AND METHODS

NCI-H295R cells (ATCC, Rockville, MD, USA) were seeded into 12-well plates (5×10^5 cells per well), maintained in DMEM/F12, 2% Ultrosor HY (Gibco, Renfrew, UK), insulin (5 μ g/ml), transferrin (5 μ g/ml), sodium selenite (5 ng/ml) (as 1% ITS, Sigma, Poole, UK) and antibiotics at 37°C with 5% CO₂-95% air. Cells were preincubated with the above growth medium containing either AII (10 nM), forskolin (10 μ M) or no agonist ('control') for 96 h, replacing medium every 24 h. After 96 h, cells were thoroughly washed with Earle's Balanced Salt solution (EBS), then treated for a further 4 h with or without AII (10 nM) at 37°C. Androstenedione, cortisol and corticosterone in the media were measured, using direct radioimmunoassays, and corrected for protein.

Results are expressed as mean Stimulation Ratios (SRs i.e. response to stimulus/basal response) \pm s.d. for n=4 experiments. Statistical tests were

performed using an unpaired, two-tailed, Student's t-test with statistical significance taken at $P < 0.05$.

RESULTS AND DISCUSSION

96 h treatment with AII caused no change in 4 h basal androstenedione production but significantly increased basal cortisol ($P < 0.05$ for 4 experiments) and corticosterone ($P < 0.05$ for 3/4 experiments). In contrast, 96 h forskolin preincubation significantly decreased 4 h basal production of all three steroids ($P < 0.05$ in 3/4 experiments) for reasons which remain unclear.

4 h treatment of NCI-H295R cells with AII (10 nM) following 96 h preincubation with AII (10 nM) resulted in an SR of $0.9(\pm 0.10)$ for androstenedione, $1.3(\pm 0.21)$ for cortisol and $1.7(\pm 0.44)$ for corticosterone. This compared with SRs of $1.7(\pm 0.64)$, $1.5(\pm 0.22)$ and $1.6(\pm 0.54)$ for androstenedione, cortisol and corticosterone respectively after 96 h preincubation with forskolin (10 μ M) (see Figure 1). If the SRs for corticosterone and androstenedione are expressed as a ratio of increased corticosterone secretion to androstenedione secretion and these ratios are taken as an indication of shift in steroid production away from adrenal androgens and towards 17-deoxysteroid secretion or vice versa, then following AII preincubation the ratio is $1.6 (\pm 0.07)$ whereas following forskolin preincubation the ratio is $1.1 (\pm 0.24)$ ($n=4$). Statistical comparison of these ratios is significant with $P < 0.02$ (see table 1).

The cells therefore produce more androstenedione in relation to corticosterone following forskolin preincubation as compared to AII preincubation. It follows that chronic forskolin treatment alters steroid production towards the adrenal androgen pathway and away from 17-deoxysteroid production, favouring a more zona fasciculata/reticularis phenotype. Chronic AII treatment markedly increases the expression of 3β HSD, P450scc and P450c18 mRNAs (2, 4). In contrast, forskolin greatly increases the expression and activity of P450c17 (3). Thus, the ratio of 3β HSD/P450c17 mRNA increases following AII treatment (favouring the

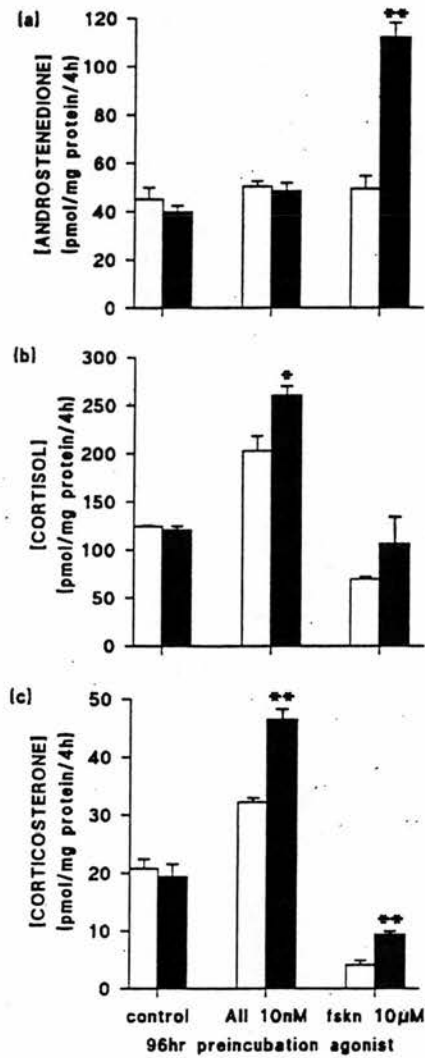


FIGURE 1

Cells were preincubated for 96 h with growth medium containing either no agonist ('control'), AII (10 nM) or forskolin (10 μM). Cells were subsequently treated for 4 h with or without AII (10 nM). These graphs show 4 h basal (□) and 4 h AII stimulated (■) secretions of androstenedione (a), cortisol (b) and corticosterone (c) following the different preincubation conditions. *P<0.05. **P<0.01. (Representative experiment of a set of four experiments).

TABLE I

Ratio of SRs (mean (+/- std dev.))			
	96h All preincubation	96h fskn preincubation	Stats. (t test)
corticosterone/androstenedione	1.6 (+/- 0.07)	1.1 (+/- 0.24)	P<0.05
cortisol/androstenedione	1.3 (+/- 0.08)	1.1 (+/- 0.45)	P>0.05
cortisol/corticosterone	0.8 (+/- 0.06)	1.1 (+/- 0.57)	p>0.05

mineralocorticoid pathway) and decreases following forskolin treatment (favouring the glucocorticoid/androgen pathway). AII is believed to exert these effects via the protein kinase C signalling pathway (4). The physiological agonist ACTH, in contrast, activates protein kinase A to mediate its effects on steroidogenesis *in vivo*. Forskolin was used to mimic this activation of protein kinase A since H295R cells show poor response to ACTH, which is possibly related to the low levels of ACTH receptor mRNA found in these cells (8).

Current theories suggest that cells in the three zones of the adrenal cortex arise from a common undifferentiated, progenitor cell because transposition of cells from all zones of the adrenal cortex to *in vitro* culture results in a loss of their zone-specific properties (9). Migration of a cell from its origin in an outer stem cell layer through all three zones of the cortex has been demonstrated by the appearance of a pattern of radial stripes of a 21-hydroxylase promoter/ β -galactosidase reporter transgene throughout the cortex of transgenic mice (10). Precisely what determines and regulates the differentiated phenotype of this cell is still unclear. The ability of the H295R cell line's steroid phenotype to be shifted from predominantly one pathway to another means that this cell line could be a useful model for studying mechanisms regulating zonation and the differential expression of the steroid pathways.

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Direct stimulation of cortisol secretion from the human NCI H295 adrenocortical cell line by vasoactive intestinal polypeptide

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Objective To investigate a possible direct action of vasoactive intestinal polypeptide (VIP) on adrenal cortisol secretion and to define its mechanism of action.

Design The human adrenocortical carcinoma cell line NCI H295, which is not contaminated by medullary chromaffin cells, was used to aid distinction between a direct action of VIP on adrenocortical cells and an indirect mechanism involving VIP-stimulated release of catecholamines.

Methods NCI H295 cells were challenged with 10^{-11} – 10^{-7} mol/l VIP for 4 h, with or without prior exposure for 72 h to 10^{-6} mol/l forskolin. Cortisol and cyclic AMP contents of the overlying media were measured using in-house radioimmunoassays. Cells were treated with 10^{-8} – 10^{-6} mol/l adrenaline or 3.3×10^{-8} mol/l VIP with and without 10^{-6} – 10^{-4} mol/l propranolol to exclude the possibility that an indirect mechanism of action involving β -adrenoceptors was operating.

Results VIP treatment produced an increase in cortisol secretion without pre-incubation, but this was markedly enhanced by prior exposure of cells to forskolin. VIP was potent, with a threshold of 10^{-11} mol/l ($n=4$), reaching a maximum 3.9 ± 0.9 -fold increase in effect on cells

pre-exposed to forskolin ($n=4$) by 3.3×10^{-8} mol/l. This increase matched the 4 h response to 10^{-6} mol/l forskolin. Cortisol secretion was accompanied by a parallel, dose-dependent increase in accumulation of cAMP.

Conclusions VIP potently and directly stimulates secretion of cortisol from these adrenocortical cells of human origin via an adenylate cyclase-coupled VIP receptor. These findings raise the possibility of a significant and direct effect of VIP in the control of steroid secretion from the adrenal cortex in humans.

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Introduction

Elevated plasma cortisol levels have been associated with high blood pressure in young people with a familial history of high blood pressure and may be an indication of a predisposition to hypertension [1]. Amongst subjects of this group there was also an increased prevalence of the AA genotype of the glucocorticoid receptor. It is possible that higher plasma cortisol levels result from the presence of this particular receptor genotype but they may equally well be an independent event. Thus, factors that increase cortisol secretion may also be involved in the pathophysiology of essential hypertension.

There is accumulating evidence to indicate that adrenocorticotrophic hormone is not the sole regulator of cortisol secretion and that neural control mechanisms influence steroidogenesis in the human and other mammalian species (for review [2,3]). The adrenal cortex of several species contains ganglion cells and nerve fibres that

synapse in close apposition to adrenocortical cells as well as blood vessels within the cortex [4,5].

Several putative neurotransmitters have been implicated as regulators of adrenal steroidogenesis, including acetylcholine, catecholamines, corticotrophin-releasing factor, neuropeptide Y and vasoactive intestinal polypeptide (VIP, for review [3]). In the case of VIP, it has been demonstrated that rat adrenal cortex contains VIP-immunoreactive nerve fibres [4] together with the VIP receptor messenger RNA being present both in the adrenal cortex and in the medulla [6]. Stimulation of the splanchnic nerve results in an increase in glucocorticoid secretion, in the presence of exogenous adrenocorticotrophic hormone, from the adrenals of conscious hypophysectomized calves [7], an effect mimicked by infusion of exogenous VIP [8]. Furthermore, stimulation of the splanchnic nerve is accompanied by the appearance of VIP in the venous effluent from conscious calves [9]. In other species, VIP

was found to stimulate androstenedione secretion from isolated, perfused porcine adrenals [10] and to increase steroid production from intact capsule-zona glomerulosa preparations from the rat [11].

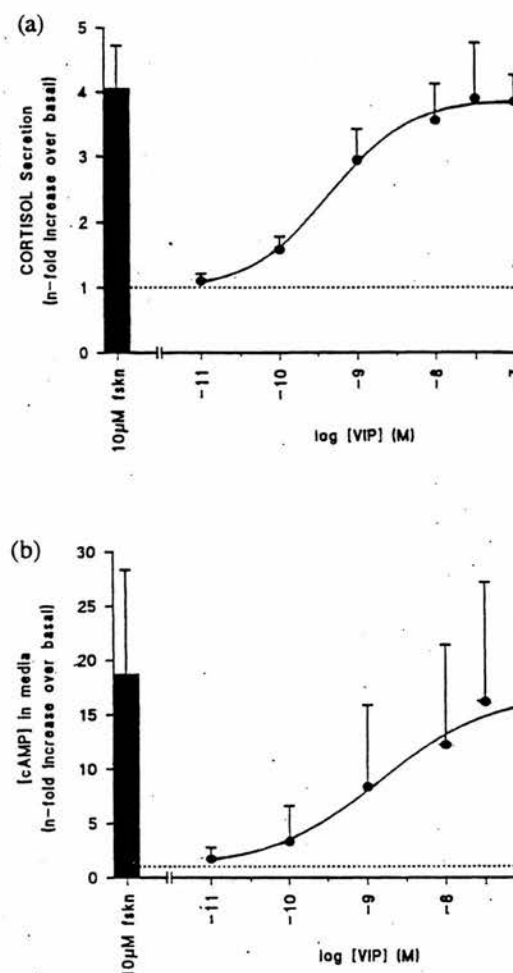
Controversy has surrounded the question of whether VIP can stimulate steroidogenesis directly. Hinson *et al.* [12] failed to obtain a response of steroid secretion to VIP in collagenase-dispersed rat adrenocortical cells but observed an increase in aldosterone secretion to VIP from an intact capsule-zona glomerulosa preparation. Because administration of VIP can stimulate release of catecholamines from medullary chromaffin cells [13], it has been postulated that it might act indirectly by releasing catecholamines from islets of chromaffin cells known to be present in the cortex [14]. The catecholamines would then stimulate secretion of steroids by acting on β -adrenoceptors present on the adrenocortical cells. In contrast, Mazzocchi *et al.* [15] reported that an increase in steroid secretion occurred with administration of VIP in dispersed rat adrenocortical cells. Because it is likely that collagenase-dispersed rat adrenocortical cell preparations could contain some contaminating medullary chromaffin cells, it is difficult to determine whether the response observed is direct, indirect or both. Bornstein *et al.* [16] reported that only an attenuation of VIP-induced secretion of steroid in response to propranolol treatment occurred in a primary culture of human adrenal cells (a mixed culture of adrenocortical and medullary cells) [16], indicating that two mechanisms of VIP action may be involved.

In order to investigate a possible direct action of VIP on adrenocortical cells, we studied the steroidogenic effects of VIP on the human adrenocortical carcinoma cell line NCI H295. This cell line was originally established from a primary invasive adrenal tumour in 1980, from a patient showing signs of mineralocorticoid, glucocorticoid and adrenal androgen excess [17]. It has since been characterized and shown to secrete steroids from the three major pathways in the adrenal cortex [18]. Because it is not contaminated by medullary chromaffin cells, the NCI H295 adrenocortical cell line is the model of choice for experiments to distinguish direct from indirect effects of VIP on adrenocortical steroidogenesis.

Materials and methods

NCI H295 cells (ATCC, Rockville, Maryland, USA) were seeded into 12-well plates (5×10^5 cells per well) and maintained in Dulbecco's modified Eagle's medium F12, 2% Ultrosor HY (Gibco, Renfrew, UK), 5 μ g/ml insulin, 5 μ g/ml transferrin and 5 ng/ml sodium selenite (as 1% ITS; Sigma, Poole, UK) at 37°C with 5% CO₂-95% air. Cells were pretreated with the above medium containing either 10 μ mol/l forskolin or no agonist ('control') for 72–96 h, replacing medium every 24 h. After pretreatment, cells were washed with Earle's balanced salt solution, incubated for 1 h in serum-free medium and finally

Fig. 1

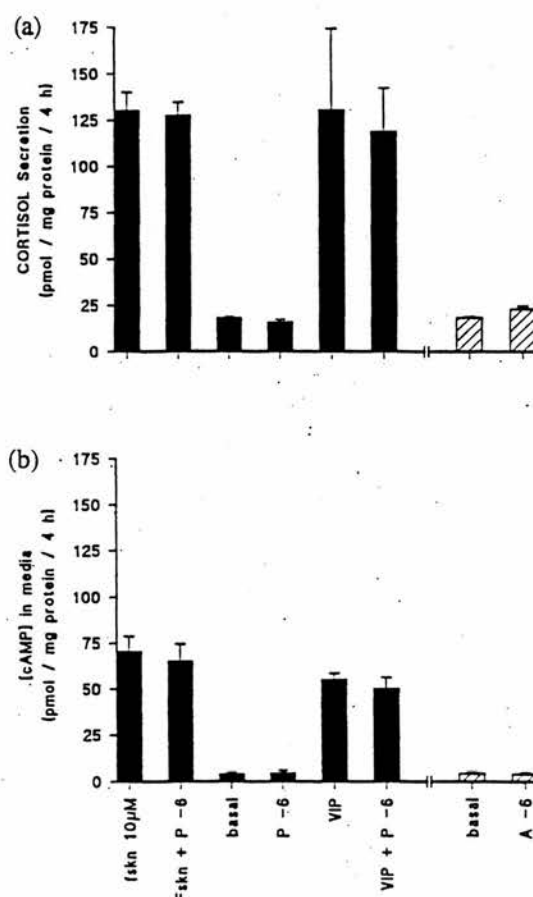


Dose-dependent increases in (a) cortisol and (b) cyclic AMP (cAMP) secretions after 4 h challenge with vasoactive intestinal peptide (VIP) (●). Maximal stimulation occurred in response to 3.3×10^{-8} mol/l VIP (a 3.9 ± 0.9 -fold increase) and was comparable to that in response to a 4 h challenge with 10 μ mol/l forskolin (fskn). Combined data (expressed as mean n -fold increases relative to basal values \pm SD) from four experiments both for cortisol and cAMP.

exposed to fresh serum-free medium with or without 10^{-11} – 10^{-7} mol/l VIP for 4 h at 37°C. For β -adrenoceptor activation studies, cells were treated in the same manner but with various doses of adrenaline, propranolol or V plus propranolol, as stated in the Results.

After 4 h, medium was removed and the cortisol and cAMP (cAMP) content measured, using direct radioimmunoassays. The cell monolayer was washed (in Eagle's

Fig. 2



Administrations of 10^{-8} , 10^{-7} and 10^{-6} mol/l propranolol (P) failed to inhibit 4 h basal, 4 h forskolin (Fskn and fskn)-stimulated ($10 \mu\text{mol/l}$) and 4 h vasoactive intestinal peptide (VIP, 3.3×10^{-8} mol/l)-stimulated cortisol (a) and cyclic AMP (cAMP, b) secretions (■; only 10^{-6} mol/l P data are shown). Representative experiment from three experiments both for cortisol and for cAMP. Four-hour challenges with 10^{-8} , 10^{-7} and 10^{-6} mol/l adrenaline (A) failed to stimulate cortisol (a) and cAMP (b) secretion above basal levels (▨; only 10^{-6} mol/l A data are shown). Representative experiment from a set of three experiments both for cortisol and for cAMP.

balanced salt solution alone) and taken up into 0.3 mol/l NaOH with 0.1% sodium dodecyl sulphate for subsequent protein determination. The steroid and cAMP measurements were corrected for cell protein concentration.

Results are expressed as means of triplicate wells \pm SD for n experiments. Statistical tests were performed using an unpaired, two-tailed, Student's t test. $P < 0.05$ was considered statistically significant.

Results

Administration of 10^{-11} – 10^{-7} mol/l VIP produced a dose dependent increase in secretion of cortisol by H295 cells. The stimulation was statistically significant for 'control' cells (no pre-incubation), in that a dose of 10^{-8} mol/l VIP resulted in a 2.0 ± 0.3 -fold stimulation of cortisol secretion relative to basal values ($n = 4$). However, this response was greatly enhanced for cells pretreated for 72 or 96 h with $10 \mu\text{mol/l}$ forskolin, for which 10^{-8} mol/l VIP elicited a 3.6 ± 0.6 -fold increase relative to basal values ($n = 4$), the threshold change occurring at 10^{-11} mol/l ($n = 4$).

The maximal response to VIP occurred at a dose of 3.3×10^{-8} mol/l both for cortisol and for cAMP production and pEC50 (negative logarithm to the base 10 of the concentration for half the maximal effect) values were 9.22 ± 0.43 ($n = 4$) and 8.57 ± 0.45 ($n = 4$) for cortisol and cAMP production, respectively (Fig. 1).

Administration of the β -adrenoceptor antagonist propranolol did not affect either basal or VIP-stimulated cortisol or cAMP secretion, which was consistent with the VIP response occurring through a specific VIP receptor and not, therefore, supportive of the hypothesis of there being an interaction of VIP with β -receptors (Fig. 2). Indeed, administration of the non-selective β -receptor agonist adrenaline failed to elicit an increase either in cortisol or in cAMP levels relative to basal values from H295 cells suggesting that there had been a loss of functional β -receptors on cells of this cell line (Fig. 2).

Discussion

The mechanism by which forskolin pretreatment enhances the subsequent response to VIP is, at present, not known. Possible explanations include upregulation of VIP receptors at the cell surface and upregulation of steroidogenic enzyme expression (since administration of forskolin markedly upregulates expression and activity of P450c17 [19]). The latter explanation is supported by the fact that pretreatment with forskolin also enhanced the response of cortisol secretion to a subsequent 4 h further administration of $10 \mu\text{mol/l}$ forskolin. The maximal steroidogenic effect of VIP was equivalent to that produced by $10 \mu\text{mol/l}$ forskolin, emphasizing that VIP is a very potent stimulus of cortisol production in these cells.

Two subtypes of the VIP receptor, VIP1 and VIP2, have so far been isolated and cloned [20,21]. Activation of VIP1 and VIP2 receptors increases intracellular cAMP levels via activation of adenylate cyclase in a variety of tissues. Administration of VIP also elicited a dose-dependent increase in cAMP production in forskolin-pretreated H295 cells which paralleled the increase in cortisol production (see Fig. 1).

In conclusion, VIP acts directly on H295 cells to stimulate steroidogenesis with an accompanying dose-dependent increase in cAMP. These findings do not rule out an indirect mechanism of action on adrenocortical cells *in vivo* but raise the possibility of an accompanying direct effect of VIP in the control of steroid secretion from the adrenal cortex of man.

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